DEVELOPMENT OF ALLOSTERIC BIOSENSORS FOR THE DIAGNOSIS OF INFECTIOUS DISEASES

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DEVELOPMENT OF ALLOSTERIC BIOSENSORS FOR THE DIAGNOSIS OF INFECTIOUS DISEASES

Memòria presentada per Rosa Mª Ferraz Colomina per optar al grau de doctor per la Universitat Autònoma de Barcelona.

Rosa Mª Ferraz Colomina

Vist-i-plau dels directors de la tesi,

Antoni Villaverde Corrales Anna Arís Giralt Rafael Cubarsí Morera

A mis padres,
a mi abuela y a David.

“Vaigues per agon vaigues, tot son costeres, així que a nesto vay per agon e mes distragut. Pel carré de San Anton capa dal, arribes agon se chunte en San Blas, allí mateix ña una roca agon se an feit demostrasions de escalada. Aixi pasan per la mateixa de esta roca, ña un caminet que te porte al costat de la Roca de la Botella. Lindiquen aixi perque te la forma de una botella, miran be a lo que represente lo seu tapado…”

Julián Naval
Corguen cam a través per la Llitera
“La Voz de La Litera”

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1. INTRODUCTION

1.1. BIOSENSORS

1.1.1. CONCEPT

According to the International Union of Pure and Applied Chemistry (IUPAC), a biosensor is “a self-contained integrated receptor-transducer device, which is capable of providing selective quantitative or semi quantitative analytical information using a biological recognition element” (Thevenot, Toth et al., 2001). The most used biological components are proteins, nucleic acids, membrane cells or tissues, whereas the signal transducers are generally microbalances, electrodes, optical components and semiconductors (Villaverde, 2003; Scheller, Wollenberger et al., 2001).

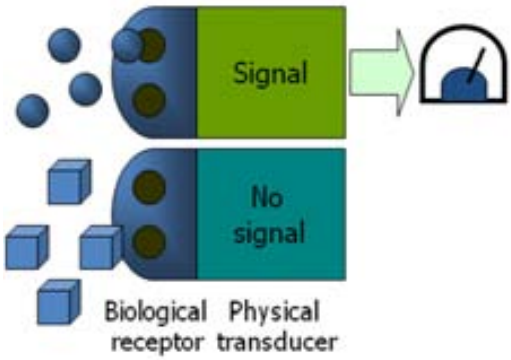

FIGURE 1. Schematic representation of a standard biosensor. The signal produced by the physical transducer is only transmitted when there is a specific recognisement between the biological receptor and the analyte. Adapted from Villaverde A (Villaverde, 2003).

1.1.2. CLASSIFICATION

Biosensors can be classified in different ways, according to the label nature of the signal (Pejcic, De Marco et al., 2006), the transducer used (Pejcic, De Marco et al., 2006; Cooper, 2003; Leonard, Hearty et al., 2007) or the general composition of the sensor (Villaverde, 2003).

1.1.2.1. LABELLING

Pejcic et al (Pejcic, De Marco et al., 2006) described a classification regarding to the labelling of the molecules:

- Labelled-type biosensors are those on which a specific label is detected. Labelled biosensors are the most used nowadays. Fluorescence-based biosensors, where a fluorescence signal is detected, or immunoassays such as enzyme-linked immunoassay (ELISA) where the analyte is detected through labelled antibodies, are clear examples of this concept.

- Non-labelled or label-free type biosensors are those sensors allowing the direct measurement of the biochemical reaction on the transducer surface without the presence of a label. Non-labelled biosensors are under continuous development and they are acquiring great interest in recent years. The biosensor signal can be measured by Surface Plasmon Resonance (SPR), which allows detection of protein-protein interactions using only refractive waves (Gauglitz and Proll, 2008; Pejcic, De Marco et al., 2006). Other important biosensing techniques are acoustic wave devices or cantilevers for detecting mass changes, infrared wave (IR) technologies, for gas analysis and detection of pollutants in marine water, or ellipsometry, a method for characterizing thin layers based on the superimposition of the reflected light from a thin layer that has been radiated with a polarized light (Gauglitz and Proll, 2008). Some of these technologies will be explained more in detail in the next chapter.

1.1.2.2. TRANSDUCER

The classification regarding the type of transducer is defined by the technology required to produce the signal. The main types of biosensors use optical and electrochemical signals. However, the use of systems based on the detection of acoustic and thermal signals is increasing.

➢ OPTICAL OR PHOTOMETRIC BIOSENSORS

Optical or photometric biosensors are those based on the reception of light that either has passed through the sample or has reflected back. The study of the electromagnetic

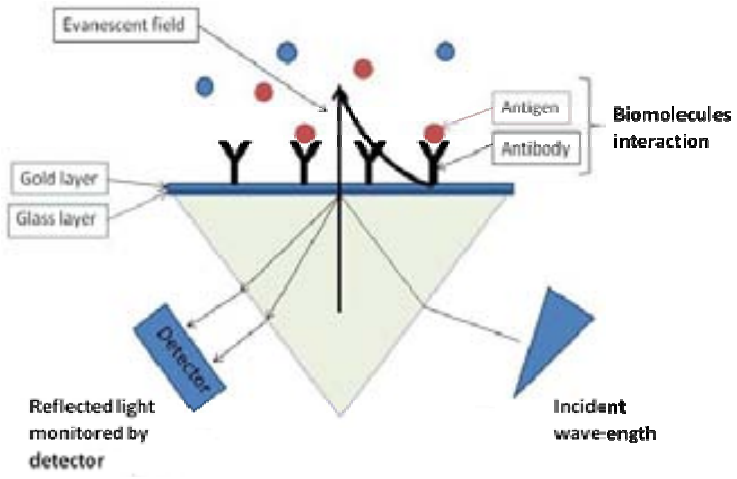

FIGURE 2. Representation of a common surface plasmon resonance system. Adapted from Habauzit et al (Habauzit, Chopineau et al., 2007).

spectrum can provide information about the changes in the environment surrounding the analyte. In 1997, Collings et al (Collings and Caruso, 1997) described the different types of optical measurement: absorption; reflection; fluorescence; chemiluminescence and phosphorescence.

Fluorescence measurements are of particular interest in biosensing because of its high sensitivity. In some immunoassays, fluorochromes are used to label molecules such as antibodies, to produce a fluorescence signal (Pejcic, De Marco et al., 2006). Nowadays it is also important the use of other techniques such as Surface Plasmon Resonance (SPR). This technology is based on the absorption of the photon energy by the free electron constellation of a thin noble metal layer (gold or silver) and its conversion into an evanescent field (Habauzit, Chopineau et al., 2007). The binding of the analyte to the receptor immobilized on the metal layer, induces a change in the refractive index of the dielectric medium, affecting the generation of the evanescent wave, and changing the incident angle (Figure 2). The change of the wavelength induced by the binding of the analyte is proportional to the quantity of retained analyte. The analyte is injected over the surface and the bound molecules can be monitored as a function of time. In the same way, the dissociation of the complex can be monitored when a buffer is injected. Therefore, this technology offers the possibility to follow in real time the binding between both molecules (Berggard, Linse et al., 2007).

Modern optical devices allowing direct, time-resolved monitoring of binding processes are low-priced, offer simple technical setups, and are user-friendly and robust (Gauglitz and Proll, 2008).

➢ ELECTROCHEMICAL BIOSENSORS

Electrochemistry is one of the most promising methods in biosensing (Gauglitz and Proll, 2008), in which the biological recognition element (biochemical receptor) is retained in direct spatial contact with an electrochemical transduction element (Thevenot, Toth et al., 2001). The sensor contains three electrodes, including one used as a reference. The signal corresponds with the derived potential from ions but subtracting that one of the reference electrode. Main advantages of this transduction are the low cost, high sensitivity, independence from solution turbidity, easily miniaturized/well suited to microfabrication, low power requirements, and relatively simple instrumentation (Guilbault, Pravda et al., 2007). These characteristics make electrochemical detection methods highly attractive for the monitoring of infectious diseases and biological warfare agents. In fact, several electrochemical approaches (*i.e.*, amperometric, potentiometric, impedance) have already been used to monitor emerging infectious diseases (Pejcic, De Marco et al., 2006).

Thevenot et al (Thevenot, Toth et al., 2001) classified electrochemical biosensors depending on their measurement mechanisms (Table 1). The method used depends on the type of change (redox, ionic, conductivity, etc) along with the properties of the analyte and matrix (Pejcic, De Marco et al., 2006). The most widespread example of a commercial biosensor is that one based on glucose oxidase enzyme (Updike and Hicks, 1967), since sugars such as glucose are detected through amperometric measurements. Glucose oxidase enzyme is able to hydrolize glucose producing gluconic acid and hydrogen peroxidase. The consumed oxygen needed for the reaction is detected by an oxygen electrode and this is converted into a measure of glucose concentration (Updike and Hicks, 1967). Another example is the detection of viruses through amperometric measurements (Los, Los et al., 2005). The basis of this system is the use of an alkaline phosphatase (AP)-catalyzed reaction that produces a redox active product detectable by an electrode. First, antibodies immobilized on a chip bind the virus particles and then, the addition of a viral-specific secondary antibody bound to the AP, allows the indirect measurement of the amount of viral particles present in the sample (Los, Los et al., 2005). In a similar way, this reaction has been used also to the detection of

oligonucleotides, but through the immobilization of molecular probes in magnetic beads (Gabig-Ciminska, Holmgren et al., 2004).

TABLE 1. Types of electrochemical biosensors. Adapted from Thevenot et al (Thevenot, Toth et al., 2001).

Measurement type	Transducer	Transducer analyte
Potentiometric	Ion-selective electrode (ISE) Gas electrode Glass electrode Metal electrode	K+, Cl-, Ca2+, F-
Amperometric	Metal or carbon electrode Chemically modified electrodes (CME)	H+, sugars, alcohols… Sugars, alcohols, phenols, oligonucleotides…
Conductometric, impedimetric	Interdigitated electrodes, metal electrode	Urea, charged species, oligonucleotides…
Ion charge or field effect	Ion-sensitive field effect transistor (ISFET), enzyme FET (ENFET)	H+, K+…

➢ PIEZOELECTRIC AND ACOUSTIC BIOSENSORS

Piezoelectric biosensors are based on the use of crystals able to suffer an elastic deformation when an electrical potential is applied. Through that potential, it is possible to obtain a characteristic resonance frequency. Thus, when analyte binds to the surface, there is a detectable change. Piezoelectric materials have become popular because of the inherent simplicity, ease-of-use, low-cost and speed to obtain the results (Uludag, Piletsky et al., 2007).

In general, acoustic biosensors are based on a type of piezoelectrical material, quartz crystal resonators. A high frequency voltage is applied to the quartz crystal to induce the crystal to resonate, and its resonance frequency is then monitored in real time. Quartz crystal resonators can be used to characterize interactions with peptides, proteins and immunoassay markers, oligonucleotides, viruses, bacteria and cells (Uludag, Piletsky et al., 2007). Acoustic systems are not affected by refractive index changes, but are instead sensitive to the viscosity and density of the media (Uludag, Li et al., 2008).

- **THERMOMETRIC BIOSENSORS**

Thermal biosensors measure changes in temperature of circulating fluid following the reaction of a suitable substrate with immobilized enzyme molecules. Thermometric biosensors have several applications such as measure of enzyme activity, fermentation monitoring, etc. However, problems related to stability and fluid handling are still avoiding the development of miniaturized micro-thermometric systems (Ramanathan and Danielsson, 2001; Ramanathan, Rank et al., 1999).

1.1.2.3. SENSOR COMPOSITION

Apart from biosensors composed by a receptor and a transducer as two different molecules or systems, protein-only biosensors are composed by a single molecule able to detect the analyte and transduce the signal (Villaverde, 2003).

1.2. PROTEIN-ONLY BIOSENSORS

Protein-only biosensors are generally enzymes including both the receptor and the transducer in the same polypeptide chain (Ferraz, Vera et al., 2006; Villaverde, 2003). The main advantage of this type of sensors is that it does not require a chemical coupling between the receptor and the signal transducer, since the whole molecule is biologically produced in a single step. Furthermore, protein engineering procedures allow the improvement and the design of new proteins with the capability to detect essentially molecules of interest in medicine and industry (Ferraz, Vera et al., 2006).

Insertional protein engineering also allows a more versatile combination of functional modules for the construction of highly responsive mosaic proteins exhibiting unusual conformational versatility upon ligand binding (Doi and Yanagawa, 1999b; Ostermeier, 2005). Obviously, a proper design of the protein is necessary to promote a good exposure of the inserted receptor to the analyte, allowing a better interaction between them. These procedures are usually based on site directed peptide insertion (Feliu and Villaverde, 1998; Hiraga, Yamagishi et al., 2004; Charbit, Ronco et al., 1991; Martineau, Guillet et al., 1992; Manoil and Bailey, 1997; Coeffier, Clement et al., 2000; Aris and Villaverde, 2004; Bckstrom, Holmgren et al., 1995) but a previous identification of the solvent-exposed permissive sites is required. The recombinant enzymes are in general stable, they can be produced in big amounts in bacterial systems, are easy to purify and its detection assay is rapid and simple (Feliu, Ferrer-Miralles et al., 2000).

According to the different peptides introduced into the protein, we can distinguish between cleavage-based platforms and allosteric platforms.

1.2.1. CLEAVAGE-BASED PLATFORMS

These biosensors are proteins displaying protease target sites. Cleavage-based platforms include proteins that can either be activated when they are cleaved by removal of a repressing domain, or inactivated (Pufall and Graves, 2002). When the protein has a fluorochrome bound to a quencher, the effect of a protease could induce the removal of the quencher allowing the observation of fluorescence (Zhang, 2004). Dual fluorescence

can also be observed if there is no interaction between two different fluorochromes of the same cleaved molecule (Blackman, Corrie et al., 2002) (Figure 3).

The insertion of specific protease target sites on the protein surface can be a good option for the development of new biosensors. If the protein is cleaved, it can be observed either by changes in its electrophoretic pattern, or in activity. It has to be taken into account that the regions close to the insert can influence peptide conformation and, as a consequence probably digestion efficiencies (Benito, Mateu et al., 1995).

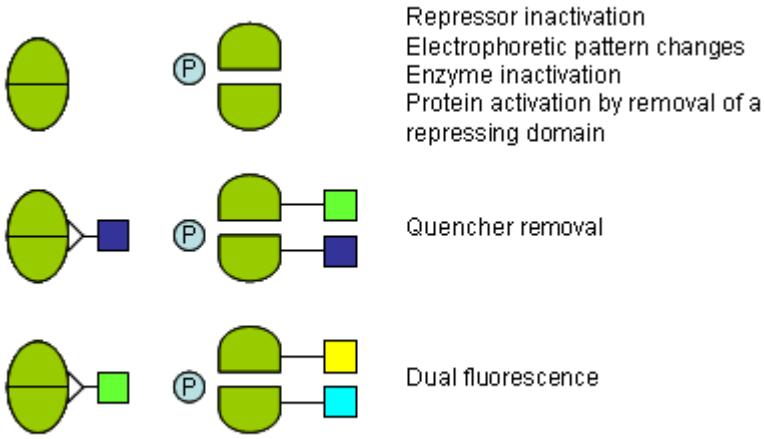

FIGURE 3. Summary of the different types of cleavage-based platforms. P: effector protease. Adapted from Ferraz et al (Ferraz, Vera et al., 2006).

It is known that many natural proteins are proteolitycally activated by the removal of self inhibitory protein domains. In fact, targeted proteolysis is a biological principle regulating many complex cellular events (Ehrmann and Clausen, 2004; Goulet and Nepveu, 2004; Hilt, 2004). In this context, Geddie et al (Geddie, O'Loughlin et al., 2005) obtained a set of p53 variants that were activated upon the regulatory element removal. This element corresponded with the carboxy-terminus fragment of p53. The cleavage was mediated by either the lethal factor (LF) or the human immunodeficiency virus (HIV) protease (Geddie, O'Loughlin et al., 2005). Moreover, protein p53 is able to interact with

specific DNA sequences, making it detectable by electrophoretic analysis (Geddie, O'Loughlin et al., 2005).

It is also common the use of fluorophors to detect the cleavage. For example, one approach is the use of monomers of rhodamine fused to the extremes of a protein containing a cleavage site for a protease. Rhodamine monomers have a high fluorescence. However, when two rhodamine monomers stack with each other and they are physically close and suitably oriented, there is a complete loss of fluorescence by self-quenching. Thus, when the protease divide the protein in two parts, separating both molecules of rhodamine, a high fluorescence signal appears (Blackman, Corrie et al., 2002). In the same way, this system can also give information about the interaction of two proteins labelled with this fluorophore (Hamman, Oleinikov et al., 1996) since the signal disappears when there is an interaction. Fluorescence also stands out when using a protein containing two molecules emitting fluorescence at different wavelengths. When a protease cleaves the protein, changes in the Fluorescence Resonance Energy Transfer (FRET) spectra are observed (Kohl, Heinze et al., 2002; Zhang, 2004).

Another example of cleavage-based platforms is the use of the cI lytic repressor of the *E.coli* lambda bacteriophage, which has been engineered to contain different target sites for proteases from HIV (Goh, Frecer et al., 2002), hepatitis C virus (HCV) (Martinez, Cabana et al., 2000) and severe acute respiratory syndrome (SARS) viruses(Martinez and Clotet, 2003). When there is a coexpression of the cI repressor and the protease, lytic lambda cycle is promoted and it is clearly observed by plaque counting. This can be used for the analysis of new protease inhibitors and also to quantitatively analyse the activity of proteases from mutant viruses emerging in patients treated with antiviral, protease-targeted drugs (Parera, Clotet et al., 2004a).

1.2.2. ALLOSTERIC PLATFORMS

In allosteric sensors, the interaction between the sensor and the analyte induces functional changes in the biosensor, generating a macroscopically detectable signal, either improving or inhibiting its activity. Allosteric platforms can be activated through the interaction with either another protein by oligomer formation, with the ligand

(Geddie, O'Loughlin et al., 2005), by proper folding of the protein (Kohn and Plaxco, 2005; Huidobro-Toro, Lorca et al., 2008) or by true allosteric modulation (Benito, Feliu et al., 1996; Ferrer-Miralles, Feliu et al., 2001; Brennan, Christianson et al., 1995; Doi and Yanagawa, 1999a). There are some enzymes on which the interaction with the ligand produces inactivation, probably by steric hindrance of the active site (Figure 4).

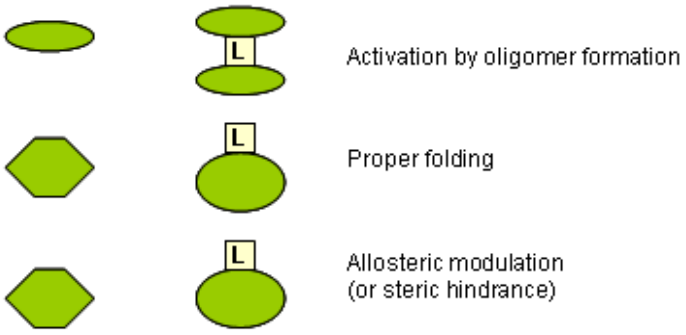

FIGURE 4. Schematic representation of allosteric platform types. L: Ligand. Adapted from Ferraz et al(Ferraz, Vera et al., 2006).

Allosterically modulated biosensors are based on the capability of a ligand to produce conformational modifications into the sensor. This property is not exclusive of proteins, since engineered ribozymes can also be allosterically activated by ATP (Breaker, 2002). All allosteric enzymes that catalyze the formation of easily detectable products are potential biosensors (Villaverde, 2003). Natural allosteric enzymes cannot be directly used as biosensors because most of their modulators are in general devoid of analytical interest. Hence, protein engineering has been of great importance to modify allosteric and non-allosteric enzymes to induce an allosteric response to new effectors by insertion of appropriate receptor sites. Therefore, it is necessary to identify the best places to accomodate the insert, which are permissive sites where the inserted segment does not disturb irreversibly the enzyme activity (Cabana, Fernandez et al., 2002; Villaverde, 2003; Webb, 2007). Several proteins such as β-galactosidase (Benito and Villaverde, 1994; Benito, Feliu et al., 1996), alkaline phosphatase (Brennan, Christianson

et al., 1995), β-lactamase (Legendre, Soumillion et al., 1999) and green fluorescent protein (GFP) (Doi and Yanagawa, 1999a) have been engineered in this way.

The main effectors of the allosteric activation are antibodies (Cabana, Fernandez et al., 2002). The use of antigenic sites as inserts into the protein-only biosensors makes possible the application of this type of sensors for infectious diseases (Benito, Feliu et al., 1996; Ferrer-Miralles, Feliu et al., 2001; Brennan, Christianson et al., 1995; Doi and Yanagawa, 1999a). Villaverde et al found that β-galactosidases containing arginine-glycine-aspartic (RGD) were activated by anti-peptide antibodies but not by RGD-targeted integrins (Feliu, Ferrer-Miralles et al., 2002; Alcala, Feliu et al., 2001). This fact indicates differences in the binding of both molecules, suggesting that antibody binding is a major force in sensor activation. Sometimes, the presence of an anti-peptide antibody inhibits the enzyme activity, such as in alkaline phosphatase and β-lactamase.

The main limitation of allosteric biosensors is the slight difference between the sensor signal and the background. Higher activation factors would be desirable for fine analytical applications where a wide dynamic range is required. However, further exploration of these sensors could improve the sensing signal to allow the proper development of efficient allosteric sensors.

1.2.2.1. β-GALACTOSIDASE

β-galactosidase from *Escherichia coli* (β-D-galactoside hydrolase, E.C. 3.2.1.23) is an enzyme involved in the heterotrophic growth of the bacteria, being the responsible for the degradation of lactose. It is also able to degrade lactose analogues such as ortho nitrophenil β-D-galactopyranoside (ONPG) or chlorophenol red β–D-galactopyranoside (CPRG), rendering a coloured product, Fluorescein β-D-galactopyranoside (FDG) producing a fluorescent signal or Galacton^{R}, with a luminescent product. This enzyme is composed by four subunits of approximately 116 KDa each (Jacobson, Zhang et al., 1994). It has been widely used as a reporter enzyme in gene expression (Casadaban, Martinez-Arias et al., 1983), as a marker for gene cloning and plasmid construction (Sambrook, Fritsch et al., 1989), as a partner in fusion proteins (Yang, Veide et al., 1995) and as a tag for process monitoring (Benito, Valero et al., 1993). Furthermore, due to its

ability to respond allosterically to antibodies, it is useful to the development of enzymatic biosensors (Villaverde, 2003). The simple quantitative enzymatic assays, tolerance to foreign polypeptide fusions and reactivation of inactive mutants are the main characteristics that make β-galactosidase useful for the design of biosensors (Feliu, Ferrer-Miralles et al., 2000). This issue will be developed later in the chapter 1.5.

1.2.2.2. ALKALINE PHOSPHATASE

Alkaline phosphatase (AP; EC 3.1.3.1) catalyzes alkaline hydrolysis of a great number of different phosphoric acid esters. It is a homodimer of 94000 Da containing $4Zn^{2+}$ and $2Mg^{2+}$ metal ions (Brennan, Christianson et al., 1995) important in catalysis and stabilization of the structure. Alkaline phosphatase is highly used as enzymatic label in immunoassay for determining herbicides, pesticides, different active organic compounds and proteins (Muginova, Zhavoronkova et al., 2007; Brennan, Christianson et al., 1995).

Insertion of an HIV peptide from envelope glycoprotein gp120 and a Hepatitis C virus (HCV) peptide into the vicinity of the active site of the alkaline phosphatase, rendered a fully active enzyme. The addition of specific anti-peptide antibodies induces an inhibition up to 40-50% of the enzyme activity (Brennan, Christianson et al., 1995). Furthermore, protein variants able to respond enzymatically to antibodies were generated by insertion of two independent point mutations. The fact that enzymatic modulation is altered from inhibition to activation by single amino acid changes in the active site of AP supports an allosteric mechanism due to structural alterations in or surrounding the active site upon antibody binding rather than the antibody mediated steric blocking of the active site (Brennan, Christianson et al., 1994).

Using three different types of alkaline phosphatases isolated from *E. coli* and intestinal alkaline phosphatases of animal origin, Muginova et al (Muginova, Zhavoronkova et al., 2007) analyzed the presence of zinc and magnesium in urea and insulin preparations using a colored compound derived from p-nitrophenyl phosphate (NPP) as an indicator of the reaction. Interference of the sample ions with Zn^{2+} and Mg^{2+} from the enzyme produced alterations in alkaline phosphatase activity, producing an inhibitory effect (Muginova, Zhavoronkova et al., 2007).

1.2.2.3. β-LACTAMASE

β-lactamases are enzymes responsible for the resistance to β-lactam antibiotics, such as penicillin and cephalosporin. They have a four atom ring called β-lactam, the origin of its name. Legendre et al (Legendre, Soumillion et al., 1999) modified genetically a β-lactamase obtaining enzymes which activity was inhibited when exposing the binding site of specific anti-prostate antigen antibodies and streptavidin. However, one of the clones they obtained (P66L4-06) responded to the antibody PSA66 enhancing its catalytic activity up to 1.7 fold (Legendre, Soumillion et al., 1999). In another work, these authors developed an engineered β-lactamase library responsive to other molecules such as horse spleen ferritin and β-galactosidase (Legendre, Vucic et al., 2002).

Binding of metal ions to allosteric sites distant from the active site of the enzyme can act also as an allosteric regulator. Mathonet et al (Mathonet, Barrios et al., 2006) described several β-lactamase mutants exposing binding sites to Zn^{2+}, Cu^{2+} and Ni^{2+} in loops remote to the active site. Metal binding induced a local conformational change and a possible broader transmission of new conformational constraints, affecting the enzyme activity. A different response was obtained depending on the ion used and on the mutant. However, in all of them an enzymatic inhibition was observed (Mathonet, Barrios et al., 2006).

1.2.2.4. GREEN FLUORESCENT PROTEIN

Green Fluorescent Protein (GFP) has a molecular mass of 26.9 KDa and it is folded as 11-stranded β-barrel that spontaneously generates its own fluorophor (Wachter, 2007). It was discovered four decades ago in the jellyfish *Aequorea Victoria* and since then, it has been used in a wide range of applications, such as reporter protein for many purposes and biosensing. It has been used, for example, for the detection of protein-protein interactions fusing two different fluorescent proteins with two proteins of interest for the measure of resultant wavelength through FRET technology (Chudakov, Lukyanov et al., 2005).

This protein was also used as an allosteric sensor by Doi N et al (Doi and Yanagawa, 1999a) who inserted a TEM-1 β-lactamase in a solvent exposed region near the

fluorophore of the protein. When the engineered GFP interacted with the β-lactamase inhibitory protein (BLIP), an increase of fluorescence without changes in the emission spectrum was observed (Doi and Yanagawa, 1999a).

In Table 2 and as a summary, there are some examples of protein-only biosensors either based on cleavage platforms or in allosteric platforms.

TABLE 2. Examples of either cleavage-based platforms or allosteric proteins obtained by insertional mutagenesis. Adapted from Ferraz et al (Ferraz, Vera et al., 2006).

Holding protein	Strategy	Insert	Analyte	Sensing mechanism	Signal (factor, when activated)	Application (proved or suggested)	References
β-galactosidase	Site directed insertion	FMDV[a] and HIV antigenic peptides	Anti-peptide antibodies and immune sera	Allosteric	Enzymatic activity up-shift	Diagnosis	(Benito, Feliu et al., 1996; Cazorla, Feliu et al., 2002; Feliu, Ferrer-Miralles et al., 2002; Feliu, Ramirez et al., 1998; Ferrer-Miralles, Feliu et al., 2001; Ferrer-Miralles, Feliu et al., 2000)
β-galactosidase	Site directed insertion	HIV protease substrate	HIV protease	Cleavage mediated inactivation	Enzymatic activity down-shift or electrophoretic analysis	Antiviral drug design and screening	(Vera, Aris et al., 2005; Baum, Bebernitz et al., 1990)
Alkaline phosphatase	Site directed insertion	HIV antigenic peptide	Anti-peptide antibodies	Probably steric hindrance	Enzymatic activity down-shift	Diagnosis	(Brennan, Christianson et al., 1994)
Alkaline phosphatase	Site directed insertion plus site directed mutagenesis of the active site	HIV and HCV antigenic peptide	Anti-peptide antibodies	Allosteric	Enzymatic activity up-shift (up to 2.5-fold)	Diagnosis	(Brennan, Christianson et al., 1995)
GFP	Site directed insertion followed by random mutagenesis	TEM1 β-lactamase	TEM1 β-lactamase inhibitor	Allosteric	Fluorescence emission up-shift (not determined)	Drug design and screening	(Doi and Yanagawa, 1999a; Doi and Yanagawa, 2002)
EGFP	Amino acid replacement	LPS/LA-binding motif	Bacterial LPS	Quenching	Fluorescence emission down-shift	Quality control (endotoxin detection)	(Goh, Frecer et al., 2002)
TEM β-lactamase	Random insertion and phage-mediated selection	Random peptides	Anti PSA antibodies	Allosteric and steric hindrance upon the specific construct	Enzymatic activity down- or up-shift (up to 1.7-fold)	Diagnosis	(Legendre, Soumillion et al., 1999)

p53	Site directed insertion plus site directed deletion	LF, HA and HSV antigenic peptides	Anti-peptide antibodies	Dimerization	Electrophoretic mobility up-shift (up to 100-fold)	Diagnosis and screening	(Geddie, O'Loughlin et al., 2005)
p53	Site directed insertion	HIV and LF protease substrates	HIV protease and LF	Auto-inhibitory domain removal	Electrophoretic mobility up-shift (up to > 100-fold) or in situ hybridisation (2-fold)	Screening	(Geddie, O'Loughlin et al., 2005)
cI lambda repressor	Site directed insertion	HIV, HCV and SARS protease substrates	HIV, HCV and SARS proteases	Cleavage mediated inactivation	Phage plaques counting (up to 50-fold)	Antiviral drug design and screening	(Martinez, Cabana et al., 2000; Martinez and Clotet, 2003; Parera, Clotet et al., 2004b)
MBP	Site directed insertion eventually followed by punctual mutagenesis	Zinc binding sites	Zinc	Allosteric	Fluorescence emission modulation (up to 8-fold)	Not specified, presumably wide	(Marvin and Hellinga, 2001)
MBP	Random insertion	TEM-1 β-lactamase segment	Maltose and other sugars	Allosteric	Enzymatic activity up-shift (up to 1.7-fold)	Not specified, presumably wide	(Guntas and Ostermeier, 2004)
DHFR	Site directed insertion eventually followed by punctual mutagenesis	FKBP macrolide-binding protein and ERα ligand binding domain	FK506 and estrogen	Binding-promoted thermostability and consequent genetic complementation	Growth of temperature-sensitive yeast under non-permissive temperatures (up to 2.5-fold)	Drug design and screening	(Tucker and Fields, 2001)
FynSH3	Deletion	none	Proline-rich peptide ligand	Ligand induced protein folding	Tryptophan fluorescence increase (up to 15-fold)	Not specified, presumably wide	(Kohn and Plaxco, 2005)
GFP-DsRed fusion	Modular fusion	TEV protease substrate	TEV protease	Cleavage mediated fluorescent tag separation	Dual fluorescent emission yield	Antiviral drug design and screening	(Kohl, Heinze et al., 2002)

1.3. BIOSENSOR APPLICATIONS

Over the last years, biosensors have become an important tool for detection of molecules in many fields. There are several examples of biosensor applications, such as detection of ions by electrochemical and optical biosensors; sugars, alcohols, phenols, urea or oligonucleotides by electrochemical biosensors (Thevenot, Toth et al., 2001; Gabig-Ciminska, Holmgren et al., 2004); interactions between peptides, proteins, immunoassay markers, oligonucleotides, viruses, bacteria and cells through acoustic (Uludag, Piletsky et al., 2007) or optical biosensors (SPR) (Berggard, Linse et al., 2007); temperature measuring in enzymatic processes such as fermentation through thermometric biosensors (Ramanathan and Danielsson, 2001); or determining presence of herbicides, pesticides, active organic compounds and proteins (Muginova, Zhavoronkova et al., 2007; Brennan, Christianson et al., 1995) by protein-only biosensors. Detection of environmental pollutants as well as benzotriazoles, dioxane and algal toxins in water (Richardson, 2007), detection of adulteration and contamination of milk and milk powder (Haasnoot, Marchesini et al., 2006), or the detection of explosives (Singh, 2007), are only some examples of other applications of biosensors. In spite of the wide range of possibilities, nowadays is becoming more and more important the development of biosensors for the detection of molecules in public health. One example is the detection of markers for cancer diagnosis. For example, Sarkar et al developed a prostate cancer marker biosensor based on an immunoassay joined to an amperometric system (Sarkar, Pal et al., 2002).

There is an urgent need, specially in developing countries, for new health-related technologies (Chin, Linder et al., 2007). In particular, early and accurate diagnosis of diseases would improve the individual health as well as that of the general public, permitting prompt and proper treatment of the patients, limiting the spread of disease in the population and minimizing the waste of public resources on ineffective treatments (Chin, Linder et al., 2007). The international community, specially the Global Fund (www.theglobalfund.org), has been addressed a strong investment to HIV/AIDS, malaria and tuberculosis treatment and diagnosis. There are other infections also important such as lower respiratory infections, diarrheal diseases (rotavirus, cholera),

childhood-cluster diseases (diphtheria, measles, pertussis, tetanus) and tropical diseases (lymphatic filiariasis, dengue, Chagas disease, leishmaniasis, guinea worm, etc.).

In table 3 is shown a summary of some of the diagnostic technologies used nowadays for several infectious diseases affecting humans and animals. Most of them correspond to the group of emerging infectious diseases, an especially important group in the last years (Pejcic, De Marco et al., 2006).

Optimal biosensor prototypes would have to work both inside and outside the laboratory to allow testing in places without qualified personnel. The concept of Lab-on-a-chip appears due to the necessity of portable devices of small size, low requirement for samples and rapid analysis (Chin, Linder et al., 2007).

TABLE 3. Examples of different used techniques to detect infectious diseases, detection limit and the reference where it is described. Modified from Pejcic et al (Pejcic, De Marco et al., 2006).

ANALYTE	BIOSENSOR FORMAT	DETECTION LIMIT*	REFERENCE
HIV	RT-PCR for HIV RNA	10 RNA copies	(Vet, Majithia et al., 1999)
	Conventional PCR	50 RNA copies	(Dube, Sherman et al., 1993; Branson, 2007)
	Immunoassays	NA	(Ausubel, Kingston et al., 1987; 2005a)
Newcastle disease	Enzyme-label immunoassay	11.1 ng ml^{-1}	(Gong, Gong et al., 2007)
	Sandwich enzyme-label immunoassay	2 ng ml^{-1}	(Lee, Thompson et al., 1993)
Forest–Spring encephalitis	Sandwich gold-label immunoassay	10^{-7} mg ml^{-1}	(Brainina, Kozitsina et al., 2003)
Hepatitis B	Methylene blue probe, PCR & DNA	NA	(Meric, Kerman et al., 2007)
	Osmium complex probe, PCR & DNA	NA	(Ju, Ye et al., 2003)
	Enzyme label immunoassay	~50 fM	(Purvis, Leonardova et al., 2003)
	Immunoassay	8 ng ml^{-1}	(Tang, Yuan et al., 2004)
	Nucleic acid	~0.01 μg ml^{-1}	(Zhou, Liu et al., 2002)
Hepatitis A	Immunoassay	NA	(Gomara, Ercilla et al., 2000)
Various toxins	Immunoassay	~ng ml^{-1}	(Rowe-Taitt, Golden et al., 2000)
Dengue	Immunoassay	NA	(Renard, Belkadi et al., 2003)
	Nucleic acid	~Picomolar	(Kwakye and Baeumner, 2003; Zaytseva, Montagna et al., 2005)
	Immunoassay	~μg ml^{-1}	(Tai, Lin et al., 2005)

***NA:** not available.

1.4. HUMAN IMMUNODEFICIENCY VIRUS: A DIAGNOSIS TARGET

1.4.1. BIOLOGY

HIV belongs to the family *Retroviridae*, group of Lentivirus. There are two types of HIV affecting humans: HIV-1 and HIV-2, but there are other viruses inside this group affecting other primates such as Simian Immunodeficiency Virus (SIV). There are also specific Lentivirus on non-primates; Maedi-Visna-Virus (VMV) in sheep, Caprine arthritis-encephalomyelitis virus (CAEV) in goat, Bovine Immunodeficiency Virus (BIV) in cattle, Feline Immunodeficiency Virus (FIV) in cat and Equine Infectious Anemia Virus (EIAV) in horse. Lentivirus virions have a size between 80-130 nm, an enveloped icosahedral capsid and two copies of +ssRNA (10Kb) (Joag, Stephens et al., 1996).

HIV is about 120 nm and its RNA is covered by a nucleocapsid composed of 2000 copies of the viral protein p24. RNA is bound to several proteins important for virus multiplication such as proteases, reverse transcriptase (RT), ribonuclease and integrase. The nucleocapsid is surrounded by a matrix composed of the protein p17 and also of the viral envelope, composed by lipids and proteins involved into the infection of new cells. These proteins are gp120 and gp41, both deriving from the polyprotein gp160 and codified by the gene *env* (2005b) (Figure 5). As detailed in table 4, HIV has in total nine genes codifying all these proteins, being the main ones *env* (envelope), *gag* (capsid and matrix) and *pol* (polymerase).

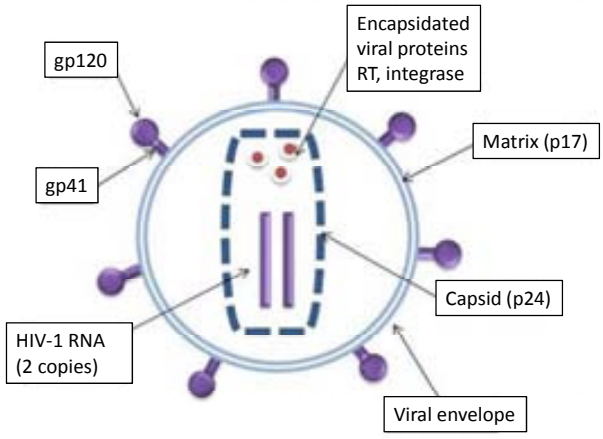

FIGURE 5. HIV virion composition. Adapted from Simon et al (Simon, Ho et al., 2006).

TABLE 4. Genes encoded in HIV genome, products and function. Adapted from Prous Science web page (http://www.ttmed.com/sinsecc.cfm?http://www.ttmed.com/sida/texto_art_long.cfm?id_dis=212&id_cou=20&id_art=1356&comecover=y&id_dis=212&id_cou=20).

Genes	Name	Product (precursor)	Viral protein	Function
Structural	***gag***	p55	p17	Myristoylated protein from viral matrix.
			p24	Main protein from viral capsid.
			p7	Nucleocapsid protein. Viral RNA packaging.
			p6	Nucleocapsid protein. Vpr binding. Viral encapsidation.
	pol	p160	p10	Protease: Gag and Gag-Pol polyprotein postranslational processing.
			p50	Reverse transcriptase.
			p15	RNase H.
			p31	Integrase.
	env	gp160	gp120	Envelope glycoprotein (surface). Viral interaction with CD4 receptor and cellular co receptors.
			gp41	Envelope glycoprotein (transmembrane). gp120 anchoring and fusion of viral and cell membrane.
Regulatory	***tat***		Tat (p14)	Transcription activator.
	rev		Rev (p19)	RNA transport between nucleous and cytoplasm.
Accesory	***nef***		Nef (p27)	Negative regulation of the presence of CD4 and MHC-I in the cellular membrane. Interference in the activation of T linfocytes. Estimulation of virion infectivity.
	vpr		Vpr (p15)	Nucleous transport of the preintegration complex. Blocking of the cellular cycle.
	vpu		Vpu (p16)	Increase of the delivering of virions into the infected cell.
	vif		Vif (p23)	Infectiosity of extracellular virions.

HIV can infect a variety of immune cells such as $CD4^+$ T cells, macrophages and microglial cells. Infection is produced through the external glycoprotein gp120 and the transmembrane protein gp41, which form the spikes on the virion's surface. Binding of these spikes to CD4 receptor and subsequent interactions between the virus and the chemokine co-receptors, generally CCR5 and CXCR4, trigger irreversible conformational changes (Simon, Ho et al., 2006). The first step after this interaction is the introduction of the viral core into the cell cytoplasm. Viral genome is then reverse transcribed into DNA by the HIV reverse transcriptase. The viral protein integrase in conjunction with host DNA repair enzymes insert the viral genome into the host chromosomal DNA. After that, virus particles can be produced using the cellular machinery in combination with virus driven transcription. Viral proteins are assembled in proximity of the cell membrane and virions expulsed through a vesicular pathway without the lysis of the cell (Figure 6).

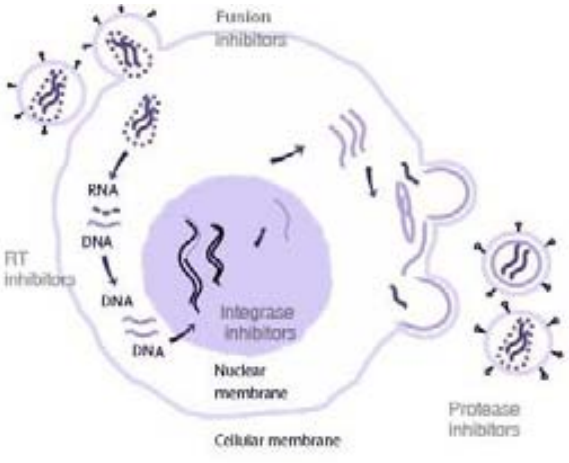

FIGURE 6. Viral replication cycle. Adapted from Simon et al(Simon, Ho et al., 2006).

1.4.2. STAGES OF HIV INFECTION

HIV infection can be divided in different stages (Alcami, 2004) (Figure 7):

1. **Primary acute HIV infection**. It can be asymptomatic or it can produce influenza or mononucleosis-like symptoms; fever, headache, pharyngitis etc. After 2 or 3 weeks the patient is totally recovered. Primary infection is characterized by high plasma viremia, low $CD4^+$ T cell and absence of HIV-1 antibodies. However, this phase is followed by a rapid immune response able to control viral load.
2. **Asymptomatic infection or clinical latency**. This stage starts after a strong immune response that reduces the number of viral particles in the blood stream. During this phase HIV is active within lymphoid organs, where numbers of virus

particles become trapped in the follicular dendritic cells (Burton, Keele et al., 2002). Along several years, the level of $CD4^+$ T cells goes down gradually. However, viral load is maintained. In fact, despite the absence of symptoms, HIV-1 is dynamically replicating along the disease course. In a chronically infected patient, the total number of virions produced can reach 1010 particles per day (Simon, Ho et al., 2006). There is a low percentage of patients maintaining the level of cells stable, who are called non-progressors patients.

3. **Symptomatic stage/AIDS**. This is the final stage of the illness. In general, AIDS appear after 10-12 years after infection, when cell mediated immunity is lost. It is characterized by a level of $CD4^+$ T cells under 200 per microliter and viral load higher than 100000 viral RNA copies per ml of plasma. At this point, several opportunistic microbes produce infections such as pneumonia, tuberculosis, candidiasis, etc. Reactivation of Herpes virus producing eruptions, Epstein-Barr induced lymphomas and even tumours can also appear. As a consequence of the lack of immune response and the opportunistic infections, the patient dies.

As it is shown in figure 7, the risk of transmission varies along infection, being more pronounced in the first and the last stages. It is also noticeable the gradual increase in viral diversity along the stages due to the high and continuous replication of the virus (Simon, Ho et al., 2006).

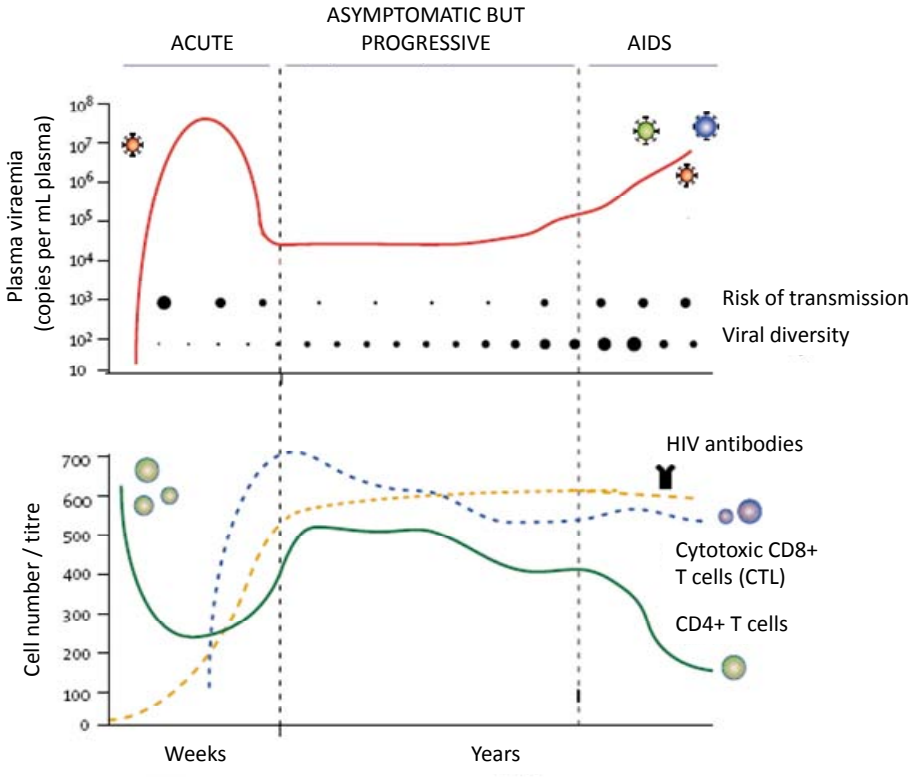

FIGURE 7. Representation of the time course of HIV infection. Plasma viremia (top) and cell number/titre (bottom) in relation to the course of infection. Adapted from Simon et al (Simon, Ho et al., 2006).

1.4.3. IMMUNE RESPONSE

1.4.3.1. CELLULAR RESPONSE

As it has been described above, immediately after infection an increase of the viral load followed by a strong response of $CD4^+$ T cells is observed. $CD4^+$ T cells are responsible for the first drop of the viral load helping B cells or cytotoxic cells, secreting lymphokines, growth factors or other facilitatory molecules. However, after a few weeks, $CD4^+$ T cells are gradually being infected and its number decreases slowly.

Antigen-specific immune responses include also cytotoxic T lymphocytes (CTL), which lyse cells expressing a foreign antigen. Although some CTLs can be $CD4^+$, normally they are $CD4^-$, $CD8^+$. CTLs are activated by the interaction with cells expressing the Major Histocompatibility Complex (MHC) type I, which are able to recognize viral proteins, internalize, process and expose them on their surface. In the case of $CD4^+$ T cells,

activation is produced through MHC type II. So, the range of epitopes to which an individual is able to respond is MHC-restricted (Coffin, Hughes et al., 1997).

During the process of the infection there is an increase of $CD8^+$ T cell titer in response to the lack of $CD4^+$ T cells. This phenomenon is called Homeostasis and is maintained until two years preceding AIDS (Margolick, Munoz et al., 1995). CTL activity is dependent in part on the help providing by $CD4^+$ T cells. For this reason, the decline in $CD8^+$ T cells can be due to the strong drop in number and functionality of $CD4^+$ T lymphocytes. Moreover, $CD8^+$ T cells rapidly respond to antigenic stimuli, generating large clones (Maini, Casorati et al., 1999).

It has been described that $CD8^+$ CTLs have a role in the regulation of the infection(Benito, Lopez et al., 2004). However, it is difficult to analyze the effects of CTLs in a laboratory due to the lack of animal models that can be infected by the virus. The most used model nowadays is *rhesus* macaques infected with SIV or chimeric HIV/SIV viruses (Ambrose, KewalRamani et al., 2007).

In 1998 Hay et al (Hay and Rosenberg, 1998) described that “nonprogressors have persistent, vigorous, virus-inhibiting CTLs whose response is broad and adaptable”. On the contrary, rapid progressors of AIDS develop a CTL response unable to adapt to viral changes (Hay and Rosenberg, 1998). Other authors observed that there is a correlation between the quality and magnitude of the CTL response in infected individuals and the rate of progression; in fact, CTL levels correlate with low viraemia levels (Cao, Qin et al., 1995).

Moreover, it has been described that treatment during primary HIV infection improves the clinical course and increases the $CD4^+$ T cell count compared to individuals who remain untreated (Kinloch-De Loes and Perrin, 1995).

Sester et al (Sester, Sester et al., 2000) developed a system to detect gag-specific CD4 and CD8 T cell responses. When the quantity of T cells in untreated patients versus treated ones was analyzed, a higher amount of $CD8^+$ T cells than $CD4^+$ T cells was found. Therefore, antiretroviral treatment induced a strong drop in the quantity of $CD8^+$ T cells (Sester, Sester et al., 2000). Lichterfeld M et al talks about the immune response

generated against viral proteins (Lichterfeld, Yu et al., 2005). Proteins inducing more strongly specific $CD8^+$ T cell response are early expressed HIV proteins, being the *nef* derived protein the most important inducer. There is also a response against other proteins produced later in the development of the infection, such as those codified by *gag* and *env* genes. Furthermore, antiviral efficiency of CTLs can vary depending of the exposed protein. It seems that proteins encoded by *gag* gene are associated with viral control during chronic phase of infection, whereas *nef* or *env* have no effect (Geldmacher, Currier et al., 2007; Heeney and Plotkin, 2006). Specific $CD4^+$ and $CD8^+$ T cell responses can be obtained when a viral lysate or gp140 is pulsed in a cell line of dendritic cells (Aline, Brand et al., 2007).

Finally, the analysis of CTLs in HIV-1 infected children just before and after treatment shows that there is a drop in HIV-specific CTLs and IFN-gamma-producing lymphocytes in patients that respond to highly active antiretroviral therapy (HAART) in comparison with non responders and non treated patients (Zhang, Zhao et al., 2006).

1.4.3.2. HUMORAL RESPONSE

Adaptative humoral response of the immune system is based on the production of antibodies by B cells. Naïve B cells are first recovered by immunoglobulins type IgM, but upon interaction with the antigen and after activation by T helper cells, there is an irreversible differentiation producing an isotype switching, which allows the production of other specific types of immunoglobulins.

Several antibody responses against HIV have been observed in recent studies. The most effective antibodies are those able to neutralize the virus, which have the ability to prevent infection of host cells by the infectious pathogen. The definition of the action and targets of neutralizing antibodies is of considerable importance for understanding the mechanisms of clinical latency and disease progression and for the development of prophylactic vaccines and immune-based therapies (Coffin, Hughes et al., 1997).

On HIV, the region inducing the majority of neutralizing antibodies is that composed by gp41-gp120 (Broliden, von Gegerfelt et al., 1992), specifically, this region comprises the variable region V3 of the surface glycoprotein gp120 (Young, Teal et al., 2004) and the

constant region C4 of gp120, responsible for the binding with the CD4+ T cell. There are also other types of neutralizing antibodies, including those recognizing V1, V2, V5, C2, and gp41 epitopes (Coffin, Hughes et al., 1997). Recent studies on the viral escape of HIV from CV-N (Cyanovirin), a carbohydrate binding agent useful as an antiviral drug, described several mutants of the virus with a major sensitivity to neutralizing antibodies. Normally, the neutralizing epitopes of the viral envelope glycoprotein gp120 are recovered by glycans, avoiding the proper recognition of these sites. The loss of glycans on the surface due to the escaping from CV-N, produce mutants on which antibodies can respond. Specifically, an increase of the neutralization sensitivity to CD4 binding site (CD4BS) antibodies, V3-loop directed antibodies and CD4-induced antibodies is observed when there is a deglycosilation of specific residues of gp120 (Hu, Mahmood et al., 2007).

Recent studies (Sheppard, Davies et al., 2007; Cavacini, Kuhrt et al., 2003) analyzed both the binding and the neutralization potency of several antibodies, normally produced in *in vitro* systems but also those derived from sera of infected patients. In this context, Sheppard et al developed a library of human monoclonal antibodies anti-HIV by means of a mouse model. These antibodies bound efficiently conformational gp41 epitopes (Sheppard, Davies et al., 2007) and one of them was weakly neutralizing. Predominant antibody isotype in sera was analyzed by Cavacini et al (Cavacini, Kuhrt et al., 2003) and a higher neutralizing activity was obtained with IgG1 immunoglobulins. Furthermore, a comparison between sera antibodies and *in vitro* produced antibodies was done, reflecting important differences between them regarding to the neutralizing ability. It was described that a human monoclonal anti CD4BS IgG3 antibody was more neutralizing than others corresponding to the isotype IgG1 (Cavacini, Emes et al., 1995). Other isotype-related HIV studies have been developed. For example, correlation between the amount of IgG2 reactive with gp41 and long-term nonprogression was observed(Ngo-Giang-Huong, Candotti et al., 2001). Khalife et al studied the isotypic response against structural proteins (*env, gag, pol*) observing a polyisotypic response against gag-products, including IgM, IgG1, IgG3 and IgA and a response to *env*-products restricted to IgG1 (Khalife, Guy et al., 1988).

Recent studies have been also focused on IgE. An inhibition effect of purified IgE from sera of HIV-infected children on the production of HIV-1 *in vitro* was observed by Pellegrino et al (Pellegrino, Bluth et al., 2002). Moreover, HIV-1 seropositive patients had significantly higher IgE levels than HIV-1 seronegative subjects, suggesting IgE as a marker of evolution of HIV disease (Miguez-Burbano, Shor-Posner et al., 1995) and an effective method to detect HIV-1 infection in adults (Fletcher, Miguez-Burbano et al., 2000). HIV-infected children with elevated IgE levels had lower IgG3 levels and higher IgG4 levels than non-infected children. As the disease progressed, HIV-1 patients failed to produce IgG3, but IgG4 antibodies remained unaffected. IgG4 antibodies have no known role in the defense against HIV-1 infection but they may increase during disease progression (de Martino, Rossi et al., 1999; Becker, 2004). Finally, a high amount of HIV-specific IgM antibodies was found early after HIV-infection (Muller and Muller, 1988; Joller-Jemelka, Joller et al., 1987).

It has been described that during the HIV infection, the first viral-directed antibodies that appear are those against the viral nucleocapsid (p24 and p17) and then those against the viral envelope (gp41 and gp120) (Lottersberger, Salvetti et al., 2003; Janvier, Baillou et al., 1991).

1.4.4. VIRAL ESCAPE

It is known that, the immunological system is not able to fight effectively against HIV infection, and this is due to the development of different strategies of the virus to escape from the immune response. In fact, apart from viral latency, viral escape and antibody neutralization are the most important determinants in the dynamics of HIV-1 infection and progression to AIDS (Wei, Decker et al., 2003).

Despite of the strong anti-HIV CTL response, which is important in the initial control and in the suppression of the viral load, the virus is able to generate mutants in important epitopes, escaping from that cells and avoiding its completely elimination (Klenerman, Wu et al., 2002). However, this is not the only viral system to escape the immune system. The virus is able to mask neutralizing epitopes of the gp41-gp120 glycan shield through the modulation of the N-linked glycosilation pattern (Wei, Decker et al., 2003;

McCaffrey, Saunders et al., 2004; Koch, Pancera et al., 2003; Hu, Mahmood et al., 2007). Hu et al demonstrated resistance to the inhibitor cyanovirin by loss of the glycan shields in the C2-C4 region of gp120 (Hu, Mahmood et al., 2007).

In the same line, the virus is able to substitute single amino acids from gp41 and gp120 to avoid the effect of inhibitors or antibody mediated neutralization. Maeda et al analyzed the regions involved in the increase of sensitivity to CXCR4 inhibitors, concluding that a single amino acid substitution from arginine to serine (R308S) in the V3 region of gp120 produced an escape mutant (Maeda, Yusa et al., 2007). This type of substitutions can also inactivate remote epitopes by long distance structural alterations that are transmitted within (Park, Vujcic et al., 1998; Park, Gorny et al., 2000; Zhang, Bouma et al., 2002; Watkins, Buge et al., 1996). Such conformational camouflage indicates a high extent of flexibility of gp41-gp120 complexes, which allows protein domains to reorganize upon receptor binding (Myszka, Sweet et al., 2000).

1.4.5. Incidence and diagnosis

According to the last report from the World Health Organization (WHO-UNAIDS), in 2007 there were estimated 33.2 million of infected people and 2.1 million people died of AIDS-related illnesses. Furthermore, there were 2.5 million of new HIV infections in 2007, with 1.7 million (68%) of these occurring in sub-Saharan Africa (Table 5) (http://data.unaids.org/pub/EPISlides/2007/2007_epiupdate_en.pdf).

The high incidence of the infection makes necessary the use of technology to detect HIV. A quick, cheap and fine detection will assure treatment and care of the patients avoiding new infections and promoting those essential resources will be used effectively by targeting ART on people who really need treatment.

TABLE 5. Data extracted from the last update of the global AIDS report 2007 (www.who.org).

	People living with HIV	New infections 2007	AIDS deaths 2007
Sub-Saharan Africa	22.5 million	1.7 million	1.6 million
South and South East Asia	4 million	340,000	270,000
East Asia	800 000	92,000	32,000
Latin America	1.6 million	100,000	58,000
North America	1.3 million	46,000	21,000
Western & Central Europe	760 000	31,000	12,000
Eastern Europe & Central Asia	1.6 million	150,000	55,000
Middle-East & North Africa	380,000	35,000	25,000
Caribbean	230,000	17,000	11,000
Oceania	75,000	14,000	1,400
Total	**33.2 million**	**2.5 million**	**2.1 million**

Nowadays, a great variety of HIV tests are available. However, due to the sophisticated equipment that some of them require, it is impossible their use in places with low resources such as Sub-Saharan Africa and South and South East Asia where there is a high HIV incidence (Table 5). For example, one of the most widely used techniques is the ELISA test (2005a). However, it is necessary to have automatic pipettes, incubators, readers, etc, and skilled technicians to operate the equipment. Ultrasensitive p24 antigen detection also requires equipment and consumables too expensive for many countries. More and more, HIV PCR tests and other nucleic acid detection techniques are being used in all over the world due to the fact that they are becoming less expensive, more automated and faster in producing results. The problem again is the necessity of equipment and personnel in these countries (1998).

Simple/rapid HIV tests are the solution for the HIV detection in laboratories with limited facilities. Advances in technology have led to the development of a wide range of these tests, such as agglutination, immunodot and immunochromatographic membrane tests. All of them are presented in a kit form, where needed reagents are included and where

the use of special equipment is not necessary. Furthermore, its use can be extended to all over the world (extracted from *Weekly epidemiological report* (1998) and http://www.who.int/hiv/topics/vct/en/index.html).

Since 2002, the Food and Drug Administration (FDA) in the United States has approved 6 different rapid HIV tests. All of them are based on the detection of anti-HIV antibodies and present sensitivities and specificities comparable to those of conventional immunosorbent assays (EIA) (table 6): OraQuick ADVANCE Rapid HIV-1/2 (OraSure Technologies, Inc., Bethlehem, PA), Uni-Gold Recombigen HIV test (Trybity Biotech, Bray, County Wicklow, Ireland), Reveal G3 Rapid HIV-1 Antibody Test (MedMira, Bayers Lake Park, Halifax, Nova Scotia), the Multispot HIV-1/HIV-2 Rapid Test (Bio-Rad laboratories, Redmond, Washington), the Clearview Complete HIV 1/2 Assay (Chembio Diagnostic Systems) and the HIV 1/2 Stat-Pak Assay (Chembio Diagnostic Systems) . In spite of being very good tests, results need to be confirmed by Western Blot or indirect immunofluorescence assays. Other important HIV test useful for the early detection of HIV infection is Aptima HIV-1 RNA qualitative assay (Gen-Probe). It is based on RNA detection and requires 4.5 hours to give a result while the rest of tests give a value between 5-20 minutes (Branson, 2007).

TABLE 6. Diagnostic characteristics of rapid HIV tests approved by the US FDA(Branson, 2007).

Assay, by specimen analyzed	Sensitivity (95%)	Specificity (95%)
Whole blood		
OraQuick ADVANCE Rapid HIV-1/2	99.6 (98.5-99.9)	100 (99.7-100)
Uni-Gold Recombigen HIV test	100 (99.5-100)	99.7 (99.0-100)
HIV 1/2 Stat-Pak Assay	99.7 (98.9-100)	99.9 (98.6-100)
Clearview Complete HIV 1/2	99.7 (98.9-100)	99.9 (98.6-100)
Serum or Plasma		
Reveal G3 Rapid HIV-1 Antibody Test	99.8 (99.2-100)	99.9 (98.6-100)
Multispot HIV-1/HIV-2 Rapid Test	100 (99.9-100)	99.9 (99.8-100)

1.5. β-GALACTOSIDASE AS A SIGNAL TRANSDUCER

1.5.1. DESCRIPTION

Protein β-galactosidase from *Escherichia coli* (β-D-galactoside hydrolase, E.C. 3.2.1.23) is an enzyme codified in *lacZ* gene and composed by four subunits of 116353 Da non-covalently bound (Jacobson, Zhang et al., 1994). It is a hydrolytic transglucosidase specific for the β-D-galactopiranoside configuration, and can act as a hydrolase as well as a transferase. Each monomer has an active site, although it needs part of the other subunits to work being only the tetrameric form active (Jacobson and Matthews, 1992). Its structure was first described by Jacobson et al in 1994 (Jacobson, Zhang et al., 1994), two years after its crystallization (Jacobson and Matthews, 1992). Each monomer is composed by five different domains and disposed on space into two axes of symmetry. Two main regions of interaction between monomers called "long interface" and "activating interface", where is located the active site, have been described. Moreover, there are several metal binding sites in β-galactosidase crystal structure. A Mg^{2+} and a Na^{+} binding site have been found into the active site of β-galactosidase and it is important to stand out that Mg^{2+} and Na^{+} are necessary for a maximal activity of the protein (Matthews, 2005).

FIGURE 8. Tridimensional structure of β-galactosidase. Deported from a RasMol representation according to the coordinates given by Jacobson et al (Jacobson, Zhang et al., 1994).

β-galactosidase is important in the metabolism of *E.coli*, being the responsible for the hydrolysis of the β-1,4 bond of the lactose, to obtain glucose and galactose, allowing the use of this sugar as a carbon source (Jacob and Monod, 1961). It also catalyzes the isomeration of lactose (galactosil-β-D-(1,4)-glucopiranose) to allolactose (galactosil-β-D-(1,6)-glucopiranose), the main inductor of *E.coli lac operon* (Huber, Kurz et al., 1976).

Furthermore, β-galactosidase hydrolyzes several lactose analogs chemically synthesized, mainly used for enzymatic assays. There are different types of substrates depending on the obtained product, being the main group the colorimetric ones. In all of them a change of colour is observed when the β-galactosidase catalyzes the reaction of hydrolysis. Ortho nitrophenil β-D-galactopyranoside (ONPG), rendering a yellow product and chlorophenol red β-D-galactopyranoside (CPRG), becoming red when it is hydrolyzed, are clear examples of this group. 5-bromo-4-chloro-3-indolil-β-D-galactopiranoside (X-gal) rendering a blue precipitate, is also important because it has been widely used in cloning processes with *E.coli* (Sambrook, Fritsch et al., 1989). Other substrates produce a fluorescent signal upon β-galactosidase cleavage, such as Fluorescein β-D-galactopyranoside (FDG) and others produce light, such as Galacton^{R}.

Even with slight modifications, β-galactosidase is a non toxic, soluble and resistant to proteolysis protein. Besides, it is easy to purify by affinity chromatography (Ullmann, 1984) and its production is easily monitored by enzymatic assays (Benito, Valero et al., 1993; Miller, 1972). Resolved tridimensional structure (Jacobson, Zhang et al., 1994) makes easier its use in cloning processes and, moreover, due to its tolerance to foreign polypeptide fusions, it is suitable to use in protein engineering procedures.

In wild type *E.coli,* β-galactosidase production is regulated by the lactose operon (*lac operon*) (Ullmann, 2001) (Figure 9). *Lac operon* is composed by structural genes, encoding for the synthesis of proteins, and regulatory genes, to control the expression of the proteins. Structural genes are *lacZ*, *lacY* and *lacA*, encoding β-galactosidase, lactose permease and thiogalactoside transacetylase respectively. Protein production is controlled by the presence of a repressor encoded by *lacI* gene, which binds to the operator to repress the transcription of lac genes. When an inducer is present, such as lactose or IPTG, the repressor is released allowing transcription of genes.

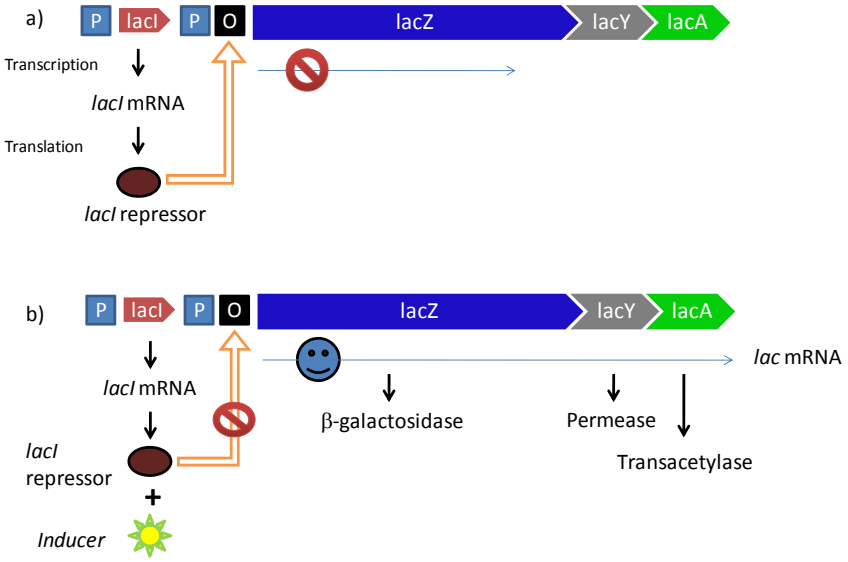

FIGURE 9. Illustration of the *lac* operon working in a repressed (a) and induced (b) state. Adapted from encyclopedia of life sciences (Ullmann, 2001).

Nowadays, a high number of commercially available vectors for constructing lacZ fusions *in vitro* are available. These vectors have been used in cloning processes using lacZ gene as a reporter gene, but also in protein production procedures to construct genetically engineered proteins (Ullmann, 2001). Expression vectors are indispensable tools to achieve a high protein production. There are several types of vectors, those on which protein production can be induced by an inductor, such as lactose or the synthetic compound Isopropyl β-D-1-thiogalactopyranoside (IPTG), and thermo-inducible vectors. The use of IPTG has been further limited in industrial applications because of its high cost and toxicity. So, thermo-inducible vectors derived from heat-sensitive *lacI* genes have appeared as a good option to avoid those problems. In these, the increase of temperature from 28 to 42ºC produces the inhibition of the repressor (Casadaban, Chou et al., 1980) and as a consequence, the expression of the protein. In general, they are based on the use of the pR and pL strong promoters of lambda phage and the cI587 repressor protein (Schauder, Blocker et al., 1987), however other efficient thermo-sensitive vectors have been described by Chao et al., who developed vector series including a $lacI^{ts}$ with different mutations and the T7 A1 promoter (Chao, Chern et al., 2002). Normally, in all of them *lacZ* deficient *E. coli* strains are used to ensure the unique

expression of the desired gene and to prevent the production of wild type β-galactosidase.

1.5.2. ALLOSTERIC PROTOTYPES

As described in the chapter 1.2.2, β-galactosidase is an enzyme with allosteric properties. Interaction with other molecules can modify its structure regulating its activity. This property and protein engineering as a tool have been used to develop biosensors that respond to antibodies directed against foot-and-mouth disease virus (FMDV) (Benito, Feliu et al., 1996; Feliu and Villaverde, 1998) and HIV (Ferrer-Miralles, Feliu et al., 2001). Antigenic peptide insertions into specific and defined solvent exposed loops from β-galactosidase resulted in modified enzymes with reduced enzymatic activity. However, in presence of specific anti-peptide antibodies, the protein increases its activity (Ferrer-Miralles, Feliu et al., 2001; Feliu, Ramirez et al., 1998). This activation occurs in an antibody titre-dependent fashion (Benito, Feliu et al., 1996). Activation factor of β-galactosidase sensors was defined as the percentage of the obtained product of the reaction in presence of antibody related to the product obtained without antibody (Ferrer-Miralles, Feliu et al., 2001).

First studies were focused to find the optimal expression system in *E.coli* for the successful production of recombinant proteins (Benito, Vidal et al., 1993). Rec A+ phenotype strains were selected due to the higher stability of the recombinant vector. It seemed that the expression of the recombinant proteins in *E. coli,* activated the RecA protein increasing the copy number of recombinant vectors (Benito, Vidal et al., 1993). Several approaches with β-galactosidase engineering early showed different effects depending on the applied modification. Changes at the N terminal end of the protein produced fewer effects into the kinetic constants than those in the C terminal end. Furthermore, insertions in several permissive sites of the β-galactosidase showed big differences in specific activity between β-galactosidase mutants (Benito, Feliu et al., 1996; Feliu, Ramirez et al., 1998). Those experiments took to the development of several protein libraries exposing antigenic sites of virus, describing the best insertion sites to have an optimal sensor response (Benito, Feliu et al., 1996; Feliu, Ramirez et al., 1998).

Three types of sensors to FMDV were described depending on the effect of the antibody binding into the Kcat/Km constants (Feliu, Ramirez et al., 1998): Those on which the binding of a specific antibody improved the substrate binding by reducing steric hindrance, those on which it improved the catalytic activity by structural changes promoted by the antibody, and finally, those on which both features were coexisting (Feliu, Ramirez et al., 1998). All these features allowed the design and development of improved enzymatic sensors.

The comparison of different types of allosteric biosensors, allowed to know if those characteristics could be extended to other proteins. β-galactosidase, alkaline phosphatase and β-lactamase mutants were analyzed altogether in order to define the molecular mechanisms for antibody-mediated modulation (Ferrer-Miralles, Feliu et al., 2000). A correlation between the activation factor of each protein and the relative Kcat (Kcat obtained for the engineered protein regarding to the Kcat obtained in the native enzyme) was done, obtaining a good correlation between them. A lower relative Kcat implied a higher activation factor. So, enzymes with lowered Kcat reached a higher activity after antibody binding than the recombinant protein alone, and enzymes with Kcat higher than the parental protein showed lower activity after antibody binding (Ferrer-Miralles, Feliu et al., 2000). Taking into account these values and the presence of several types of proteins, it was concluded that the theoretical upper limit of reactivation of any protein is close to 200%. As different proteins were included, this value seems to represent a common limit in the enzyme flexibility of different proteins. Theoretically, there is an impossibility to construct a β-galactosidase insertional mutant with a Kcat higher than 2 fold that of the unmodified protein, representing a limitation in sensitivity of analytical systems (Ferrer-Miralles, Feliu et al., 2000).

Several studies were then developed trying to find the conditions responsible for a better activation of allosteric proteins. Cazorla et al combined different concentrations of both enzyme and substrate and arrived to values of 500% of activation using a model of β-galactosidase with a single FMDV insertion (Cazorla, Feliu et al., 2002). Multiple peptide insertions in β-galactosidase were also studied. Residues 134-156 from VP1 capsid protein of FMDV were inserted in several sites of β-galactosidase, exposing 1, 2 or

3 copies per monomer. Antibody-mediated activation factors are higher in β-galactosidase sensors with 8 insertions than others with only one per monomer (Cazorla, Feliu et al., 2002; Feliu, Ferrer-Miralles et al., 2002). However, when 12 copies of the same peptide are exposed, the protein became instable enough to avoid the proper effect of the antibody. Despite the limitation in the number of insertions; the increase in the number of insertions in the β-galactosidase from 1 to 2 per monomer seems to result in a global improvement of epitope presentation and antibody binding. Moreover, stability and specific activity of the enzyme are decreased.

In further approaches, several β-galactosidase proteins including the main immunogenic segments of the protein gp41 (namely P1 and P2 epitopes) from the envelope of HIV were developed (Ferrer-Miralles, Feliu et al., 2001). Different sized peptides comprising amino acids 579-618 of the precursor gp160 were inserted in two acceptor sites (position 795 and 278) of β-galactosidase. In all of them, thermo-inducible vectors expressing lacZ gene under the control of the pR and pL promoters of lambda phage and the thermosensitive repressor cI587^{ts} (Casadaban, Chou et al., 1980) were used. These proteins were also characterized through calculation of Km, Kcat and kcat/Km, and the peptide size was described as a critical point to a better activation factor (Ferrer-Miralles, Feliu et al., 2001). Kcat and Km were strongly affected by peptide insertion in an apparently opposite fashion. Moreover, Km value increased depending on the peptide size (Ferrer-Miralles, Feliu et al., 2001; Ferrer-Miralles, Feliu et al., 2000).

Among all those proteins, the best response was obtained to the protein NF795gpC. This protein is composed by residues 579 to 613 from the polyprotein gp160, precursor of gp41, including the linear epitope P1 and only a fragment of the conformational epitope P2. Analysis of P1 surface accessibility through a model revealed a bigger exposure of this protein than the others (Ferrer-Miralles, Feliu et al., 2001).

Despite of the high variability of recombinant proteins developed, the maximum activation values obtained by the binding of an anti-peptide antibody were 250%, similarly to the previous upper limit obtained.

2. OBJECTIVES

The general aim of this work was the development and characterization of protein-only biosensors for the detection of infectious diseases, using Human Immunodeficiency Virus as a model and protein engineering as the technique for recombinant protein development. The specific aims that have guided work in this thesis are:

1. Characterization of the previously developed HIV biosensor NF795gpC. Obtainment of the optimal concentrations of protein and substrate to obtain a better sensing response.

2. Study of the parameters of NF795gpC activation depending on the substrate used and selection of the optimal substrate regarding sensitivity, signal-background ratio, range of response and sensibility.

3. Evaluation of the immunoreactivity and level of allosteric response of the protein NF795gpC testing different types of antibodies, either monoclonal or polyclonal.

4. Functional characterization of the main isotypes of sera antibody subpopulations responsible for the activation of the protein.

5. Immobilization of the biosensor into a surface and analysis of performance of the immobilized biosensor.

6. Analysis of the effect produced by the antiretroviral treatment in the composition and function of antibodies activating the sensor.

7. Development of new biosensors exposing other antigenic sites of HIV to improve the sensitivity of the test.

3. RESULTS

3.1. PUBLICATION I

PROFILING THE ALLOSTERIC RESPONSE OF AN ENGINEERED β-GALACTOSIDASE TO ITS EFFECTOR, ANTI-HIV ANTIBODY

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SUMMARY

A previously developed recombinant β-galactosidase called NF795gpC showed a good exposure of the inserted peptide on the enzyme surface and among a series of related biosensor prototypes, it was the best responder in presence of specific anti-peptide antibodies (Ferrer-Miralles, Feliu et al., 2001). Maximum activation values obtained were of 250% with an amount of protein NF795gpC of two pmol and a concentration of 2mg/ml of ONPG. Here, and similarly to the previous work with FMDV-based sensors(Cazorla, Feliu et al., 2002) a wide range of substrate and enzyme concentrations were tested in an attempt to improve the sensor signal adjusting the assay conditions. Topographical maps of the enzymatic activity and activation factors were built. The analysis of the plots gave the optimal concentrations of both protein and substrate to obtain a better signal:background ratio, that reached values up to 4-fold. Different concentrations of a specific anti-peptide antibody were also explored, showing that the antibody was not the topographical definer but the enzyme and substrate concentrations. Moreover, the use of a secondary antibody suggested that multivalent antibody binding and the consequent enzyme network formation enhance allosteric activation.

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Profiling the allosteric response of an engineered β-galactosidase to its effector, anti-HIV antibody

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Abstract

Escherichia coli β-galactosidase responds enzymatically to antiviral antibodies when a viral antigenic peptide, acting as receptor, is conveniently displayed in the vicinity of the active site. The allosteric response of a β-galactosidase molecular sensor containing a B-cell epitope from HIV has been finely dissected upon binding of an effector monoclonal antibody, within a wide range of standard concentrations of both enzyme and substrate. The topography of the enzymatic activation reveals a wide set of conditions in which the enzymatic response renders a signal over threefold the background, that is suitable for analytical biosensing. Moreover, at discrete enzyme–substrate coordinates, the effector antibody promotes an enhanced activation factor up to fivefold. The insertion of the 37-mer viral peptide between β-galactosidase residues 795 and 796 is observed as inducer of the structural flexibility required for molecular sensing, whose dynamics and efficiency are intimately associated with the concentrations of enzyme and substrate, the two partners in the signal transduction event.

Keywords: Antibody; Allosteric enzyme; Beta-galactosidase; Biosensor; HIV; Signal transduction

Biosensors are chemical sensors in which the recognition system uses a biochemical mechanism [1]. They are hybrid devices consisting of a receptor site for analyte binding and recognition, usually cells or cell components like proteins, nucleic acids or membranes, and a physicochemical transducer of diverse natures [2]. On the other hand, allosteric enzyme activation is based on protein structural flexibility required for a molecular signal to be transmitted from the effector-binding site to the active site. This property has been exploited to construct compact molecular biosensors that respond enzymatically to effectors relevant in diagnosis, especially antibodies, in a concentration-dependent fashion. The adaptive antibody binding and the conformational fluctuations occurring in the paratope can be then transformed into an analytically useful signal (substrate production) by the transducer component of the biosensor (the active site).

The engineering and re-engineering of both allosteric and non-allosteric enzymes [3] by directed point mutation [4], insertional mutagenesis [5,6] or directed molecular evolution [7] has resulted in interesting prototypes, working satisfactorily in fast homogeneous assays with sensitivity levels comparable to those of conventional diagnostic tests [8,9]. This parameter and others such as discrimination, resolution or stability could be improved by further protein engineering and by adjusting the conditions for the biosensing reaction and signal transduction. However, the allosteric mechanics is not completely understood from its enzymatic side, and this fact prevents a rational approach to construct such optimised biosensors. To gain insights into the nature of the sensing event in allosteric devices, we have explored here the dynamics of the enzymatic response of a modified β-galactosidase in front of an effector, anti-human immunodeficiency virus (HIV) antibody, by scanning the generated signal (namely the ratio of the product formed in presence and in absence of effector) in a wide range of substrate and enzyme concentrations. This method offers topographical maps

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of the enzymatic sensing that permit a fine analysis of the enzyme and substrate as key partner elements for the conversion of a conformational stimulus into an enzymatic signal.

Materials and methods

Proteins and protein purification. Protein NF795gpC is a modified *Escherichia coli* β-galactosidase in which a 37-mer HIV-1 peptide has been accommodated into a solvent-exposed loop, in the vicinity of the active site [8]. The resulting protein exhibits then one copy of the viral peptide per monomer and a total of four copies per enzyme tetramer, which is the active form of the enzyme. The foreign stretch includes the immunodominant B-cell epitope P1 of the gp41 structural protein, spanning amino acids 579–613 of the Env precursor. Binding of both polyclonal and monoclonal anti-peptide antibodies to this peptide on the enzyme stimulates the hydrolysis rate of lactose analogues rendering coloured compounds, which act as a biosensing useful signal. Protein NF795gpC was produced in *E. coli* BL26 as directed from the temperature sensitive expression vector pNF795gpC and purified from crude cell extracts by affinity chromatography as described [10]. The harvested protein was dialysed against Z buffer [11] and quantified both spectrophotometrically at 280 nm and by Bradford analysis as indicated [8]. Molar values given in the figures refer to the 116 kDa NF795gpC monomers.

ELISA and β-galactosidase activity assay. A conventional, indirect enzyme-linked immunoassay (ELISA) was performed by using 0.65 pmol/well of NF795gpC as bound antigen, with different concentrations of a mouse, anti-P1 monoclonal antibody (mAb). Details about second antibody and standard developing procedures are provided elsewhere [8]. Activation assays were performed in ELISA microplates according to previously described procedures [8]. Briefly, different concentrations of NF795gpC in buffer Z were mixed with the effector antibody in the same buffer and incubated for 60 min at 25 °C in a microtitre plate Labsystems IEMS reader, in a total volume of 80 μl. Then, 40 μl of *ortho*-nitrophenyl β-D-galactopyranoside (ONPG) at different concentrations was added and the reaction was carried out for 3 h at 25 °C. Measures of absorbance were automatically taken every 10 min. Every microassay for a specific set of conditions was performed at least in duplicate (most of them in triplicate), with standard deviations always lower than 10% of the obtained data. Individual panels shown in Fig. 3 represent the average activation values for every enzyme–substrate value pair, and any of them has been constructed from up to 12 ELISA plates with a total of 1100 independent reactions. Data from these plates were combined and plotted by using SigmaPlot 2000 v 6.0. The spectra of ONPG concentrations monitored for the *X*-axis were 1.66, 3.32, 4.97, 6.6, 8.3, 10, and 11.6 mM. For the *Y*-axis, the concentrations of sensor usually employed in the reactions were from 1.25 to 18.75 nM, measured at intervals of 1.25 nM. As indicated, some experiments were performed up to 25 nM. A second, anti-mouse mAb (IgG) from BioRad was used for some experiments at a dilution 1:2000 and in a molar excess respecting the first, anti-HIV mAb. In that cases, the first effector antibody was incubated with the enzyme for 60 min and the second antibody for another period of 60 min. Controls for these experiments without the second antibody were performed by incubating the enzyme and the effector antibody for 120 min.

Results

Binding of the effector antibody and enzyme up-modulation

The binding of the effector, anti-P1 monoclonal antibody to protein NF795gpC was monitored by ELISA and plotted for comparison along with the accompanying enzymatic response (Fig. 1). In both cases, the dose-dependence of the signal was excellent and the linearity was lost by saturation between 5 and 15 ng/μl of antibody. The antibody concentration rendering 50% of the maximum binding signal in ELISA was 0.4 ng/μl, and a value slightly higher (around 0.8 ng/μl) was required to promote 50% of maximum activity increase in the biosensing assay. This difference is compatible with the observation that the simultaneous binding of effector antibodies to more than one monomer significantly enhances the enzymatic response of the sensor [12]. This result also confirms the allosteric nature of the biosensing signal of NF795gpC and the antibody binding as the unique effector for the generation of the enzymatic signal.

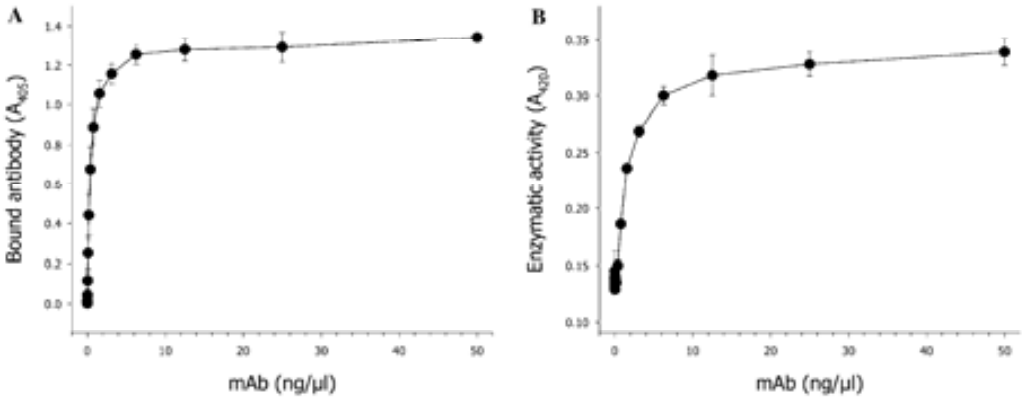

Fig. 1. Antibody binding and sensor activation. Anti-P1 antibody binding to NF795gpC, as measured by indirect ELISA (A). For comparison, NF795gpC activation measured as the amount of product formed, as promoted by the same antibody amounts (B). The activity in absence of antibody indicates the background of the assay.

Kinetics of enzyme up-modulation

The response of NF795gpC was kinetically analysed as activated by 12.5 ng/μl mAb, a concentration close to the saturation values. We wondered if the molar ratio between active site and substrate, in allosteric β-galactosidases being the unique components of the physicochemical transducer, could determine the efficiency of the signal transduction. After a first screening of different ranges for both partners, we selected 7.5 and 16.25 nM NF795gpC and 3.32 and 10 mM ONPG, respectively, as pair values close to the standard Miller assay for β-galactosidase determination [11] and in which the resulting enzymatic signals were initially found to be high. The activation events were monitored for more than 3 h and the results are plotted in Fig. 2. After 10 min of reaction, the enzyme in absence of antibody was not further processing substrate and the amount of formed product remained low and stable. Contrarily, in presence of the effector antibody, the product of the ONPG hydrolysis increased under both conditions with a slightly different kinetics, rendering a signal:background ratio (namely activation factor) between three- and fivefold. A minor but also slightly differential fading of the signal and concomitantly of the activation factor was observed according to previous reports [9], what was probably due to degradation of the yellow product of the ONPG hydrolysis occurring after long incubation periods. The maximum activation factors were reached between 30 and 50 min, after a rapid increase tending to a plateau from about to 20 min on.

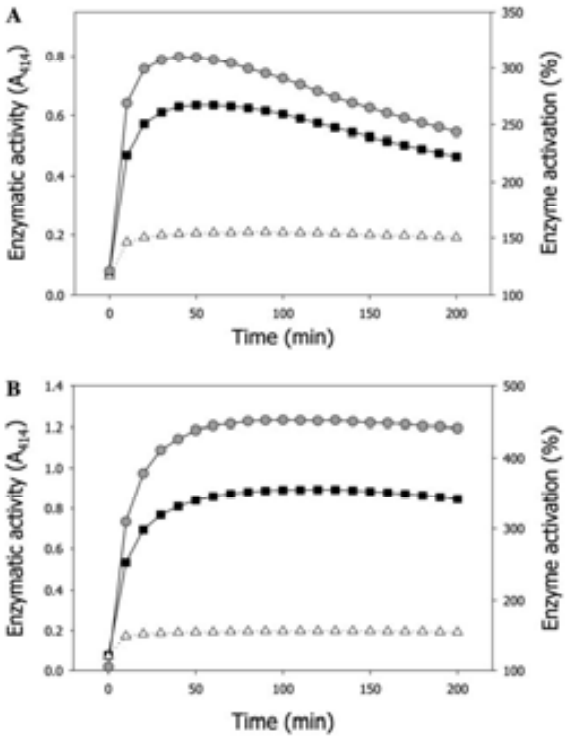

Fig. 2. Kinetics of sensor activation under different conditions. Dynamics of product formed by NF795gpC activity in absence (white triangles, left scale) and in presence (black squares, left scale) of anti-P1 monoclonal antibody, under different enzyme and substrate concentrations, namely 7.5 nM NF795gpC and 3.32 mM ONPG (A), and 16.25 nM NF795gpC and 10 mM ONPG, respectively (B). The activation factor, as a quotient of both values, is shown by grey circles (right scale).

Interestingly, the activation factors reached in these experiments were higher than those observed experimentally in previous studies, and also higher than that predicted as the maximum range of allosteric signal (roughly twofold) by comparison of diverse prototypes [13]. This fact proved that the allosteric sensors are more flexible than that previously assumed and that the activation value suggested as the upper limit was probably limited by specific reaction conditions that might be critical in the signal transduction process. In this context, the different activation factors reached in the two biosensing analysis (Fig. 2) revealed a noticeable variability in the allosteric performance. This fact prompted us to investigate the biosensor activation in a wider spectrum of enzyme and substrate concentrations to identify conditions that could be eventually more convenient for an enhanced response.

Topography of the allosteric response

The activation of NF795gpC was scanned under different enzyme:substrate combinations by sampling each axis at short intervals, thus generating topographical maps of the allosteric response. By this analysis, an important sensing area was observed with signal values over threefold (defined by the yellow sector in Figs. 3A–C) that might be very much appreciated for diagnostic applications. Interestingly, this highly responsive region was not homogeneous and showed an irregular topography in which the activation factor peaked at discrete coordinates, up to values close to fivefold. The coordinates of these enhanced sensing peaks did not occur in an entirely concentration-dependent pattern for any of the two surface axes, although a weight of both partners' concentration and specially of ONPG was clearly observed. Also, some hot positions were already defined at early times of the reaction (Fig. 3A) and they remained nearly stable throughout the substrate hydrolysis. Consequently, the general profile of the map was essentially conserved (Figs. 3A–C). Interestingly, higher enzyme concentrations (namely 20 and 25 nM) were not supportive of very efficient antibody sensing, only rendering moderate activation values (see legend of Fig. 3). Original data showing product concentration in absence and in

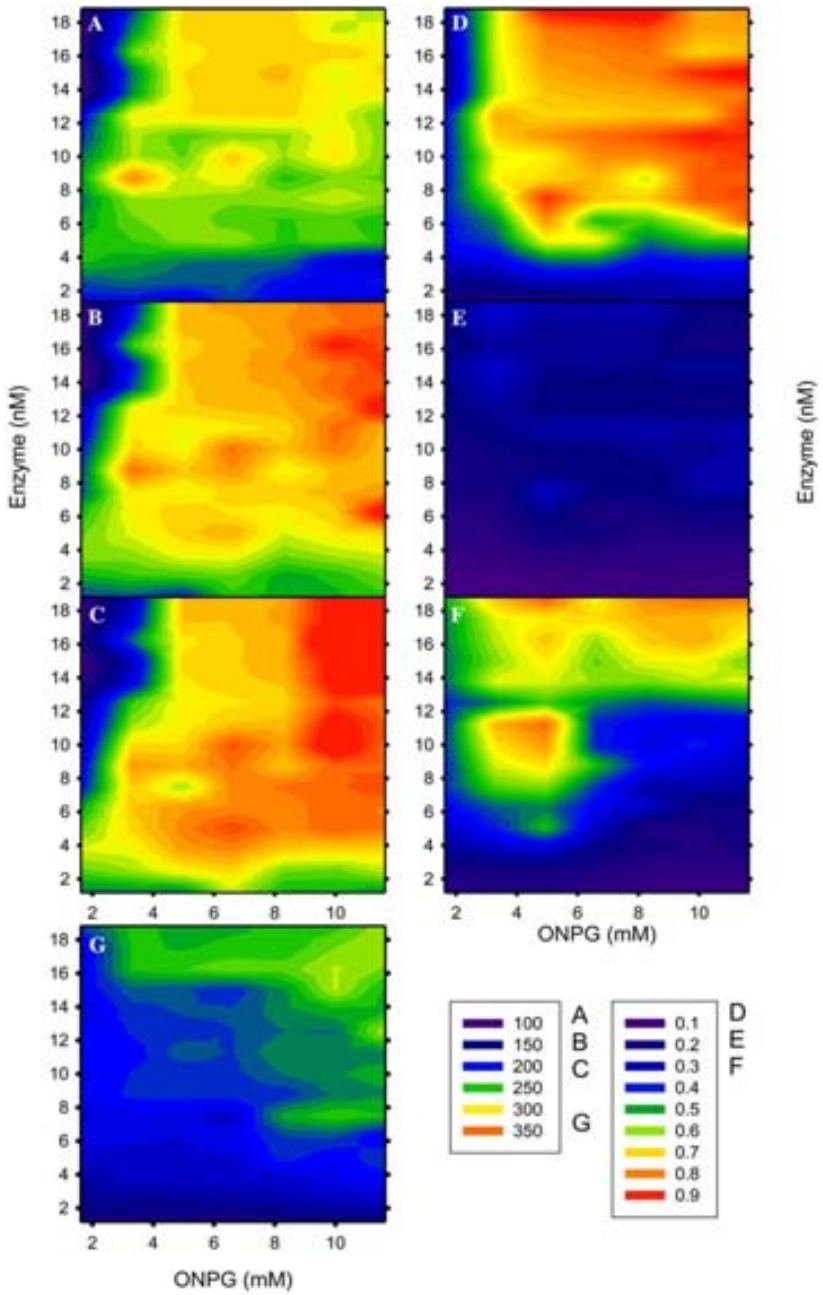

Fig. 3. Topography of NF795gpC biosensing reaction. Topography of the biosensing reaction of NF795gpC as triggered by 12.5 ng/μl (83 nM) mAb anti-P1 antibody, in a wide range of substrate and enzyme concentrations. The activation factor is shown at different times after the addition of the substrate, namely 10 (A), 20 (B), and 50 min (C). Activation factors were also calculated with higher concentrations of NF795gpC. At 20 min and for 1.6, 3.3, 4.9, 6.6, 8.3, 10.0, and 11.6 mM ONPG, they were 287.9%, 307.4%, 317.7%, 311.1%, 313.0%, 325.7%, and 300.1% (at 21.25 nM), and 285.1%, 334.2%, 307.3%, 313.4%, 318.8%, 332.1%, and 317.5% (at 25 nM), respectively. Also, rough data (formed product) from which (B) has been generated is shown in panels (D) (in presence of antibody) and (E) (in absence of antibody). Product formed by a wild type *E. coli* β-galactosidase are shown in (F). In this case, depicted data are from a shorter reaction time (3 min) to avoid saturation and for a better comparison with (E). Note that the specific activity of NF795gpC is around 20% of that of unmodified β-galactosidase [8]. The activation map of NF795gpC as mediated by 1.56 ng/μl (10 nM) mAb is also shown after 20 min of the addition of substrate (G). It must be noted that for comparison purposes, the scale for activation has been fixed up to 350% for all the displayed panels, what might partially eclipse top activation values. As an example, activation factors up to 395% (at 11.6 mM ONPG and 6.25 nM enzyme) and up to 438% (at 10 mM ONPG and 16.25 nM enzyme) were obtained in (B,C), respectively.

Table 1
Modulation of the NF795gpC activation factor by a second, anti-effector antibody

Anti-P1 mAb (nM)	ONPG (mM)	Protein concentration (nM)					
		3.3	(1.2; 10.9)[a]	5.0	(0.8; 7.2)	7.7	(0.5; 4.6)
		−Anti-mAb	+Anti-mAb	−Anti-mAb	+Anti-mAb	−Anti-mAb	+Anti-mAb
4	3.3	165.53 ± 4.50[b]	182.92 ± 4.23	173.47 ± 1.39	196.29 ± 2.65	187.24 ± 1.62	200 ± 1.57
	10.0	165.94 ± 3.68	186.14 ± 1.62	174.81 ± 5.57	200.47 ± 6.34	173.17 ± 7.53	214.63 ± 1.51
36	3.3	211.03 ± 3.35	212.29 ± 4.99	231.18 ± 2.12	231.57 ± 5.48	236.76 ± 6.63	241.7 ± 21.70
	10.0	214.18 ± 4.62	229.34 ± 11.09	238.19 ± 5.10	239.11 ± 7.90	238.42 ± 2.45	249.6 ± 7.17

[a] Beside protein concentration, molar mAb:enzyme ratios are expressed in brackets for the two concentrations of effector antibody, from lowest to highest.
[b] Activation values are given as percentage plus standard deviation.

presence of the antibody are shown in Figs. 3D and E, respectively, after 20 min of reaction. The ratio between both panels generated the activation map depicted in Fig. 3B. The topography of wild type β-galactosidase activity was also included as a control (Fig. 3F), to compare with that of NF795gpC activity in absence of the effector antibody and to evaluate the impact of the peptide accommodation of the enzyme performance.

From all these data, the concentrations of both enzyme and substrate, the two partner molecules involved in the biosensing signal transduction, were revealed as critical for generating enhanced biosensing signals. To evaluate the possible weight of the effector concentration on the activation topography, an additional experiment was performed using the poorly activating mAb effector concentration of 1.5 ng/μl (Fig. 1B). Despite the lower activation values, the topographical map occurred in a similar pattern to that observed with high mAb concentration (compare Figs. 3B and G), the top signal zones being already visible with less effector molecules. This fact excludes the antibody concentration as a topographical definer and confirms enzyme and substrate concentrations as major controllers of the allosteric profile.

Antibody–enzyme ratio and enzyme activation

High protein concentrations supported activation factors significantly higher than threefold (Figs. 3A–C). However, over 18.75 nM, NF795gpC was not activated beyond this level (see legend of Fig. 3). At 18.75 nM, the resulting mAb:enzyme monomer ratio was about 4.4 and it declined concomitantly at higher values. Therefore, a molar excess of mAb might stimulate the allosteric signal, in agreement with the enhanced activation observed when more than one effector molecule binds the tetrameric enzyme [12]. However, a molar excess of the effector, being a bivalent ligand, would also promote oligomeric complexes by cross-linking the enzyme. To evaluate if complex formation, irrespective of multiple contacts in a single enzyme molecule, could also modulate the allosteric signal, we incorporated a second anti-mAb antibody to the activation reactions at different ratios of the effector antibody:enzyme monomer. Interestingly, the second antibody enhanced the activation factor under different conditions, but only when the ratio between the first, effector antibody, and the enzyme occurred below 4.5 (Table 1). Indeed, this suggested that this enhancement occurred by inducing the formation of complexes, and that at about 4.5 mAb molecules per enzyme, such complexes already occurred spontaneously. To further explore the suggested dependence of the signal enhancement and the ratio between the effector antibody and the enzyme, we performed an additional, comparative experiment. In this analysis, the positive impact of the second antibody was evident at effector:enzyme monomer ratios between 0.2 and 1.3, but it was still observable up to 4.5 (Fig. 4). Furthermore, a nested ANOVA confirmed both the significant difference between the two sets of signals

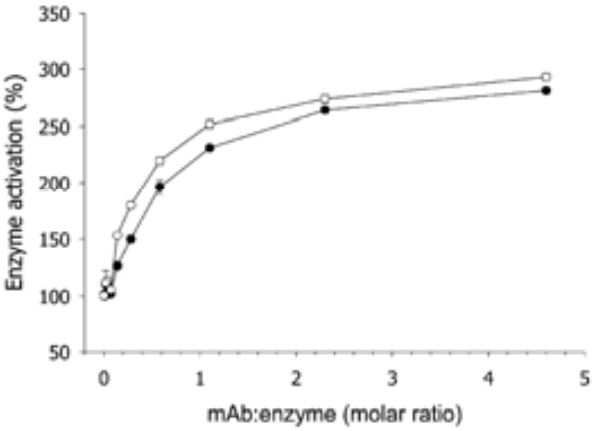

Fig. 4. Enhanced activation by a second, anti-effector antibody. NF795gpC activation has been monitored at different ratios of the effector, anti-P1 mAb in absence (black symbols) and in presence (white symbols) of a second, anti-mouse mAb (IgG). The concentration of NF795gpC was 7.7 nM and that of the anti-P1 mAb ranged from 0 to 36 nM. ONPG was used at 10 mM. Note that the second antibody alone did not affect the activity of the enzyme in absence of the effector antibody.

shown in Fig. 4 ($p < 0.001$), and also, that the enhancement of the signal was dependent on the concentration of the first antibody ($p < 0.001$).

Discussion

Allosteric enzymes undergo effector-mediated modulation through remote conformational effects, which are transmitted from the effector-binding site to the active site. An important extent of structural flexibility is then required, that in non-allosteric enzymes can be gained either by point [4,7] or insertational [5,6] mutagenesis. The antibody adaptive binding (or induced fit) efficiently triggers allosteric responses in enzymes engineered to act as molecular biosensors [3], what has permitted the generation of new diagnostic prototypes based on variations of the specific activity. The activity of *E. coli* β-galactosidase is naturally modulated by Mg^{2+} [14], by thiol reagents [15], and also by antibodies raised against the enzyme [16], proving a structurally responsible architecture with potential for biosensing. From a series of insertional mutants carrying antigenic peptides, two tolerant insertion sites were identified between residues 279–280 and 795–796, in which the epitope-carrying stretches act as allosteric receptors [6,8,13]. Both accommodation sites are within solvent-exposed, unstructured, and flexible loops, either protruding from one monomer to other forming the activating interface, or sited apart from the active site and monomer–monomer contact regions, respectively [17,18].

Therefore, in NF795gpC, the effector antibody promotes activation by long-distance transmission of conformational changes. Although some oligomeric enzymes respond allosterically through modifications in the association–dissociation constants [19,20], antibody binding to NF975gpC does not influence enzyme tetramerisation [8]. When determining the activation factor in a range of enzyme and substrate concentrations, an irregular landscape was observed (Figs. 3A–C), with a wide area over threefold activation factor at high concentrations of both enzyme and substrate. The intensity of this signal is sufficient for efficient antibody detection in new-generation diagnostic assays based on allosteric biosensing, which were previously confined to enzyme and substrate concentrations only allowing a twofold factor [8,13]. Activation up to fivefold also occurred in the upper margins of this sector within 12.5 and 18.75 nM enzyme and 8 and 12 nM ONPG (namely high-concentration sector). However, discrete coordinates within 5 and 12.5 nM enzyme and 4 and 10 nM ONPG (low-concentration sector) also supported highly efficient biosensing. This heterogeneous activation pattern, unnoticed up to now, reveals that the allosteric response is amplified at defined concentrations of enzyme and substrate (not restricted to a sole substrate:enzyme molar ratio) that allow a high divergence between the product formed ratios in absence and presence of the effector antibody. The concentration of the effector is not influencing the signal transduction topography (Fig. 3G), for which enzyme and substrate have been identified as key elements. Interestingly, the peptide insertion in β-galactosidase to render NF795gpC did not dramatically affect the activity profile of the enzyme (compare maps in Figs. 3E and F), but it would have instead enhanced the molecular flexibility required for signal transduction and also provided an allosteric-binding site for molecular sensing in a sensitive surface of the enzyme.

Finally, the fine exploration of the biosensing mechanics strongly suggests that multivalent antibody binding and the consequent enzyme network formation enhance allosteric activation (Table 1 and Fig. 4), this event being clearly observable at antibody:enzyme ratios over 4.5 (Table 1). At this value and especially at lower ratios, a high-molar excess of second anti-effector antibody significantly amplify the allosteric signal (Table 1 and Fig. 4). This occurs without modifying the tetramerisation efficiency [8] (note that *E. coli* β-galactosidase dimers are not active), and probably by modulating the adaptive binding to the receptor site and the consequent conformational signal. This fact contributes to explain why, at exception of the β-lactamase inhibitory protein BLIP [7], antibodies but not other ligands such as integrins [21,22] have been observed as excellent effectors for allosteric biosensors based on different enzymes [4–6,9], and it prompts the further adaptation and exploitation of these nanoinstruments for fast molecular diagnosis of infectious diseases.

Acknowledgments

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References

[1] D.R. Thévenot, K. Toth, R.A. Durst, G.S. Wilson, Electrochemical biosensors: recommended definitions and classification, Biosens. Bioelectron. 16 (2001) 121–131.
[2] A. Huber, S. Demartis, D. Neri, The use of biosensor technology for the engineering of antibodies and enzymes, J. Mol. Recogn. 12 (1999) 198–216.
[3] A. Villaverde, Allosteric enzymes as biosensors for molecular diagnosis, FEBS Lett. 554 (2003) 169–172.
[4] C. Brennan, K. Christianson, T. Surowy, W. Mandecki, Modulation of enzyme activity by antibody binding to an alkaline phosphatase-epitope hybrid protein, Protein Eng. 7 (1994) 509–514.
[5] D. Legendre, P. Soumillion, J. Fastrez, Engineering a regulatable enzyme for homogeneous immunoassays, Nat. Biotechnol. 17 (1999) 67–72.

[6] A. Benito, J.X. Feliu, A. Villaverde, β-Galactosidase enzymatic activity as a molecular probe to detect specific antibodies, J. Biol. Chem. 271 (1996) 21251–21256.
[7] N. Doi, H. Yanagawa, Evolutionary design of generic green fluorescent protein biosensors, Methods Mol. Biol. 183 (2002) 49–55.
[8] N. Ferrer-Miralles, J.X. Feliu, S. Vandevuer, A. Muller, J. Cabrera-Crespo, I. Ortmans, F. Hoffmann, D. Cazorla, U. Rinas, M. Prevost, A. Villaverde, Engineering regulatable *E. coli* β-galactosidases as biosensors for anti-HIV antibody detection in human sera, J. Biol. Chem. 276 (2001) 40087–40095.
[9] J.X. Feliu, N. Ferrer-Miralles, E. Blanco, D. Cazorla, F. Sobrino, A. Villaverde, Enhanced response to antibody binding in engineered β-galactosidase enzymatic sensors, Biochem. Biophys. Acta 1596 (2002) 212–224.
[10] A. Ullmann, One-step purification of hybrid proteins which have beta-galactosidase activity, Gene 29 (1984) 27–31.
[11] J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972.
[12] P. Alcalá, N. Ferrer-Miralles, A. Villaverde, Co-activation of antibody-responsive, enzymatic sensors by a recombinant scFv antibody fragment produced in *E. coli*, Biotechnol. Lett. 24 (2002) 1543–1551.
[13] N. Ferrer-Miralles, J.X. Feliu, A. Villaverde, Molecular mechanisms for antibody-mediated modulation of peptide-displaying enzyme sensors, Biochem. Biophys. Res. Commun. 275 (2000) 360–364.
[14] M. Martinez-Bilbao, R.E. Huber, The activation of beta-galactosidase (*E. coli*) by Mg(2+) at lower pH values, Biochem. Cell Biol. 74 (1996) 295–298.
[15] R. Golan, U. Zehavi, M. Naim, A. Patchornik, P. Smirnoff, M. Herchman, Photoreversible modulators of *Escherichia coli* beta-galactosidase. 1-Benzoyl-1-cyano-2-(4,5-dimethoxy-2-nitrophenyl)-ethene and 1,1-dicyano-2-(4,5-dimethoxy-2-nitrophenyl)-ethene, J. Protein Chem. 19 (2000) 123–128.
[16] F. Celada, R. Strom, Beta-galactosidase: immune recognition of conformation and mechanism of antibody-induced catalytic activation, Biopolymers 22 (1983) 465–473.
[17] R.H. Jacobson, X.J. Zhang, R.F. DuBose, B.W. Matthews, Three-dimensional structure of beta-galactosidase from *E. coli*, Nature 369 (1994) 761–766.
[18] J.X. Feliu, A. Villaverde, Engineering of solvent-exposed loops in *Escherichia coli* β-galactosidase, FEBS Lett. 434 (1998) 23–27.
[19] T.W. Traut, Dissociation of enzyme oligomers: a mechanism for allosteric regulation, Crit. Rev. Biochem. Mol. Biol. 29 (1994) 125–163.
[20] T.W. Traut, P.A. Ropp, A. Poma, Purine nucleoside phosphorylase: allosteric regulation of a dissociating enzyme, Adv. Exp. Med. Biol. B 309 (1991) 177–180.
[21] J.X. Feliu, A. Benito, B. Oliva, X. Avilés, A. Villaverde, Conformational flexibility in a highly mobile protein loop of foot-and-mouth disease virus: distinct structural requirements for integrin and antibody binding, J. Mol. Biol. 283 (1998) 331–338.
[22] P. Alcalá, J.X. Feliu, A. Aris, A. Villaverde, Efficient accommodation of recombinant, foot-and-mouth disease virus RGD peptides to cell-surface integrins, Biochem. Biophys. Res. Commun. 285 (2001) 201–206.

3.2. PUBLICATION II

ENHANCED MOLECULAR RECOGNITION SIGNAL IN ALLOSTERIC BIOSENSING BY PROPER SUBSTRATE SELECTION

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SUMMARY

This paper explored the effect of six different β-galactosidase substrates regarding the activation and allosteric profile of the protein NF795gpC. The substrates X-gal and lactose were discarded in the first assays. In the first case, the instability of the enzyme in the buffer required for X-gal dissolution unabled to perform the assay. On the other hand, lactose, the natural substrate of the protein, rendered glucose concentrations in absence of antibody under the detection limits in a standard amperometric detection system. Thus, a comparative sensing analysis between the remaining substrates (ONPG, CPRG, FDG and Galacton^{R}) was done, obtaining the parameters that define sensor activation profile in each case. Emission of light and fluorescence, although being themselves highly sensitive signals, did not result into detection of lower analyte concentrations. However, colorimetric substrates showed optimal features for the activation assays. ONPG offered the lower analyte detection limit and CPRG the higher activation values and the wider activation range. These results prompted us to use CPRG for colorimetric sensing of specific antibodies in plasma samples for further assays.

COMMUNICATION TO THE EDITOR

Enhanced Molecular Recognition Signal in Allosteric Biosensing by Proper Substrate Selection

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Abstract: Among protein biosensors, those based on enzymatic responses to specific analytes offer convenient instruments for fast and ultra-fast molecular diagnosis, through the comparative analysis of the product formed in presence and in absence of the effector. We have explored here the performance of five β-galactosidase substrates during the activation of a β-galactosidase sensor by antibodies against the human immunodeficiency virus (HIV). Interestingly, the employed substrate determines the dynamic range of the allosteric signal and significantly influences the sensitivity of the senso-enzymatic reaction. While ortho-nitrophenyl β-D-galactopyranoside allows the detection of a model anti-gp41 monoclonal antibody below 0.024 ng/μL, phenol red β-D-galactopyranoside offers the most dynamic response with signal/background ratios higher than 12-fold and a detection limit around 0.071 ng/μL. The hydrolysis of both chromogenic substrates generates linear sensing responses to immune human sera and parallel time-course topologies of the allosteric reaction. Therefore, the obtained results stress the potential of chromogenic substrates versus those rendering quimioluminescent, amperometric, or fluorescent signals, for the further automatization, miniaturization, or adaptation of β-galactosidase-based biosensing to high-throughput applications.

Keywords: antibody; β-galactosidase; *E. coli*; HIV; molecular diagnosis

INTRODUCTION

Among the different types of protein sensors under current development, allosteric biosensors are those based on an engineered reporter enzyme that responds to the analyte binding by detectable changes in its activity, either inhibition or desirably up-modulation (Villaverde, 2003). Allosteric biosensors are especially useful for diagnosis of infectious diseases, since antibodies have been observed as the most effective effectors. This fact can be accounted by the adaptive antibody binding (alternatively named induced fit), which having a conformational impact on the interacting epitope it is further transmitted to functional areas of the holding enzyme. In these sensors, the antibody-binding peptide is displayed in the vicinity of the active site, accommodated in permissive sites of the enzyme. By this approach, less active variants are generated that are eventually up modulated by immune sera (Ferrer-Miralles et al., 2000). Enzyme activation occurs in a titer-dependent fashion (Benito et al., 1996) and within a dynamic range particular for specific protein constructs. In general, the insertion sites for epitope display have been identified by trial-and-error insertional mutagenesis, but a few examples of directed evolution are also available (Doi and Yanagawa, 2002). Alkaline phosphatase (Brennan et al., 1995), green fluorescence protein (Doi and Yanagawa, 1999), β-lactamase (Legendre et al., 1999), β-galactosidase (Benito et al., 1996), and certain proteases (Saghatelian et al., 2003) and ribozymes (Ferguson et al., 2004; Sekella et al., 2002) have been engineered according to this principle generating interesting prototypes. In our laboratory, we have produced several β-galactosidase-based allosteric sensors that are successfully activated by sera either from foot-and-mouth disease virus (FMDV)-infected animals (Feliu et al., 2002) or from human immunodeficiency virus (HIV)-infected humans (Ferrer-Miralles et al., 2001).

The robustness of these β-galactosidase biosensor prototypes could be noticeably increased by enhancing the signal/background ratio, which under standard conditions is not much higher than twofold (Ferrer-Miralles et al., 2001). This could be achieved by protein engineering but also by tailoring the sensing reaction and improving the product detection, for which the selection of an appropriate substrate could be critical. Since the *E. coli* β-galactosidase has been extensively used as a marker in molecular biology for rather different analytic purposes, a diversity of analogues of lactose, the natural substrate of the enzyme, has been

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developed for specific assay requirements. Five of these substrates plus lactose were included in a comparative analysis of human, anti-HIV antibody sensing. Interestingly, the sensitivity, dynamic range of response and other properties of the assay have been observed as dramatically influenced by the employed substrate. Particularly, the proper use of chromogenic compounds results in highly enhanced signal/background rations over 12-fold and in detection limits below 0.024 ng/μL for a model monoclonal antibody and below 1/38,000 for a model sera pool. These results are discussed in the context of the potential of allosteric β-galactosidases in high-throughput molecular diagnosis.

MATERIALS AND METHODS

Proteins, Protein Production, and Purification Procedures

Protein NF795gpC is a modified *E. coli* β-galactosidase in which a 37-mer HIV-1 peptide has been accommodated into a solvent-exposed loop, in the vicinity of the active site (Ferrer-Miralles et al., 2001). The resulting protein displays one copy of the viral peptide per monomer and a total of four copies per enzyme tetramer, the active form of the enzyme. The foreign stretch includes the immunodominant B-cell epitope P1 from the gp41 structural protein, spanning amino acids 579–613 of the Env precursor. Binding of both polyclonal and monoclonal anti-peptide antibodies to this peptide, as displayed by the enzyme, stimulates the hydrolysis rate of lactose analogues (Ferrer-Miralles et al., 2001). Protein NF795gpC was produced in *E. coli* BL26 as directed from the temperature sensitive expression vector pNF795gpC, and purified from crude cell extracts by affinity chromatography (Ullmann, 1984). The harvested protein was dialyzed against Z buffer (Miller, 1972) and quantified both spectrophotometrically at 280 nm and by Bradford analysis as indicated (Sambrook et al., 1989). Molar values given in the figures refer to the 116 kDa NF795gpC monomers.

Enzymatic Assays

The sensing enzymatic assays were performed in ELISA microplates according to previously described procedures (Feliu et al., 1998). Briefly, different concentrations of NF795gpC in buffer Z were mixed with the effector antibody in the same buffer and incubated for 60 min at 25°C in a microtiter plate Labsystems IEMS reader, in a total volume of 80 μL. Then, 40 μL of chromogenic substrates ortho-nitrophenyl β-D-galactopyranoside (ONPG) or chlorophenol red β-D-galactopyranoside (CPRG) at different concentrations were added, and the reaction was monitored for 3 h at 25°C. Measures of absorbance were automatically taken every 10 min at 414 nm for ONPG and 540 nm for CPRG. 5-Bromo-4-chloro-3-indol-β-D-galactoside (X-gal) was also used for some experiments prepared as described (Sambrook et al., 1989).

To plot titration curves, 0.65 pmols of the sensing enzyme were exposed as indicated above, to different concentrations of antibody or sera and also to either chromogenic (ONPG or CPRG), fluorescent (β-D-galactopyranoside (fluorescein di), FDG) and luminescent (Galacton^R) substrates. The final concentration was 1.10 mM in all cases except Galacton^R that was added to 0.008 mM.

Activation assays for FDG were performed in Fluoro-Nunc^{TM} black plates, special indicated for fluorescence-based assays. The assay was performed as previously described with the chromogenic substrates but in this case using a VICTOR^{3}V 1420 multilabel counter from PerkinElmer. The samples were excited at 485 nm and the fluorescence emission was measured at 535 nm. Activation assays using the luminescence substrate Galacton were performed in B&W isoplates from Wallac and they also were monitored by VICTOR^{3}V 1420 multilabel counter from PerkinElmer after adding 60 μL of 0.2 N NaOH to stop the reaction.

Glucose produced during the hydrolysis of lactose was determined by a standard blood glucose meter (GLUCOCARD MEMORY 2, from A. Menarini diagnostics), which detects the intensity of the electric signal generated during the oxidation of potassium ferrocyanide. The reaction was stopped 20 min after adding lactose by boiling the mixture for 10 min, and an aliquot used to soak the assay strip as indicated by the manufacturer for blood samples. Alternatively we used the colorimetric Glucose assay kit from SIGMA, based on the rising of the brown-colored, oxidized form of *o*-dianiside. Briefly, protein NF795gpC in buffer Z was mixed with the effector antibody in a final volume of 80 μL for 1 h at 25°C. After adding 160 μL of the assay reagent and 40 μL of lactose, the mixture was incubated 30 min at 37°C, and the absorbance further measured at 450 nm.

The given substrate, enzyme, and antibody concentrations are referred to the final assay volume corresponding to 120 μL in all cases except in the glucose analysis using Glucose Assay kit from SIGMA that corresponds to 280 μL.

Sera and Antibodies

A monoclonal antibody (mAb) against the immunodominant P1 epitope within HIV gp41 was used for most of the assays (Ferrer-Miralles et al., 2001). Also, human HIV-immune sera (generously provided by Dr Miguel Angel Martínez from Hospital Universitari Germans Trias i Pujol) and a pool of 36 immune human sera were used for some experiments. Sera dilutions given in the figures refer to the aliquot of 20 μL added to the sensing reaction.

RESULTS

Substrate Selection for β-galactosidase-based Enzyme Sensors

To explore the potential of different substrates in improving the antibody sensing process by engineered β-galactosidases,

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we comparatively analyzed the performance of the prototype protein sensor NF795gpC upon its activation by anti-HIV antibodies. Initially, we selected the three commonly used chromogenic lactose analogue substrates ONPG, CPRG, and X-gal, plus FDG and Galacton that once hydrolyzed by the enzyme render fluorescent and luminescent products, respectively. Lactose, the natural substrate of the enzyme was also included in the analysis, since one of its hydrolysis products, glucose, can be efficiently determined by colorimetric but also amperometric assays.

In a first substrate screening, NF795gpC was unstable in the buffer required for X-gal dissolution (not shown), and this substrate was then discarded for further analysis. On the other hand, the use of lactose to monitor antibody sensing was firstly tested for a model anti-P1 mAb by using a personal glucose blood tester. Since human blood contains glucose, we tried to optimize glucose production to overcome those basal levels, by screening different substrate and enzyme concentrations around those previously seen as optimal for other substrates (Ferraz et al., 2004). At 9.1 nM NF795gpC and 3.3 mM lactose, 55 nM effector anti-P1 mAb produced 57.6 ± 13.3 mg/dL of glucose, and slightly lower values at different concentrations of enzyme and substrate (not shown). Since the glucose produced in absence of the antibody, if any, was under the detection limits, precise sensor activation values (regarded as the relative increase of activity mediated by the antibody) were not obtained. The alternative use of the colorimetric Glucose assay kit from Sigma, (at 3.55 nM NF795gpC, 1.43 mM lactose, and 23 nM anti-P1 antibody), allowed determining the produced glucose in absence of effector and therefore solving the factor in which the antibody mediated activation (182.6%). This activation value was very similar to that initially described for ONPG during the characterization of β-galactosidase-based sensors (Benito et al., 1996), but again the obtained concentrations of glucose were below those expected to be found in sera. In this context, determination of anti-HIV antibodies in human immune sera rendered no detectable activation values (not shown). Therefore, lactose, although technically useful for β-galactosidase allosteric biosensing was also discarded as substrate for further analytical purposes that might involve mammalian sera.

Comparative Sensing Analysis Based on Chromogenic, Luminogenic, and Fluorometric Substrates

The profile of allosteric activation was then compared by using four substrates, among those initially intended, which seemed potentially applicable to animal sera samples. Therefore, the response of the enzyme to the effector anti-P1 mAb was plotted against analyte concentration. The resulting, best-fitting rectangular equation

$$y = y_0 + a * x/(b + x)$$

in which x represents the antibody concentration, y the sensor activation factor, y_0 the basal activity, and a and b correspond with the maximal activation factor and the apparent dissociation constant for the effector antibody bound to the enzyme, respectively, was adjusted to cover the theoretically expected pairwise value 0–100 (no activation in absence of analyte). As observed (Fig. 1), the general activation profile resulted significantly influenced by the substrate used to determine enzymatic activation and the obtained equation constants were clearly distinguishable between substrates (Table I). The activation of the sensor was linear at low effector concentration values but such dependence was modified at higher concentrations. To identify the inflexion points, indicative of analyte concentrations in which the sensing capability is saturated, we plotted pairs of converging regression lines for each plot from Figure 1 and the crossing points identified and shown in Table I (r^2 values for these line sets were ranging from 0.839 to 0.999; not shown). Interestingly, the sensing signal remained saturated at

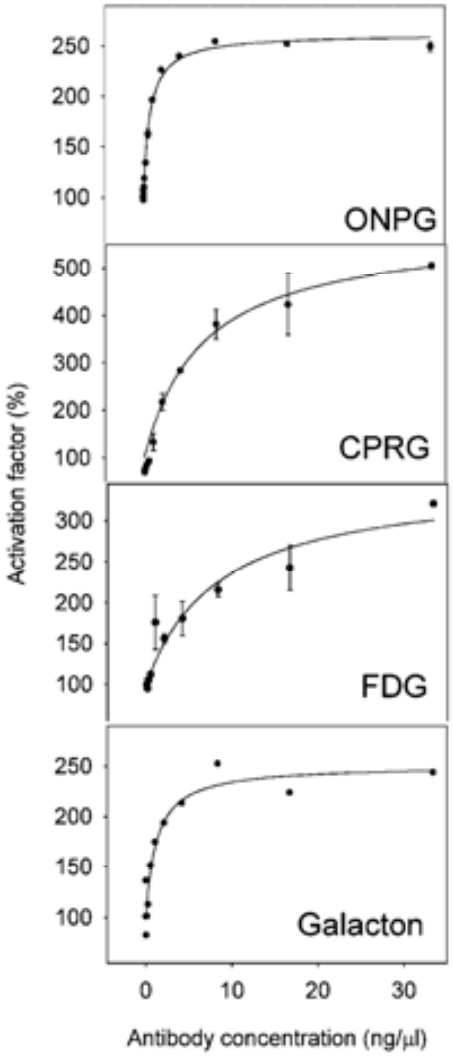

Figure 1. Enzymatic activation of NF795gpC upon exposure to anti-HIV monoclonal antibody as determined by the hydrolysis of different substrates.

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between 1 and 2 ng/μL mAb when using either Galacton or ONPG but the activation was still progressive above this value for FDG (up to about 2.7 ng/μL) and CPRG (up to about 5.2 ng/μL). In particular, CPRG showed an excellent linear response from 8 to 33 ng/μL effector mAb ($r^2 = 0.999$), with activation factors over 500% (while they hardly reached 300% when using other substrates).

Regarding the lower detection limit, ONPG allowed the most sensitive assay response, since with this substrate, the protein sensor detected up to 0.024 ng/μL specific antibody with an activation factor of 105% (threshold up-shift considered of analytical value) (Table I). With CPRG, instead, the sensor generated a detectable signal from 0.071 ng/μL, while Galacton and FDG allowed a lower detection limit in between those of chromogenic substrates (0.04 and 0.16 ng/μL, respectively). In this context, it should be noted that parameters from rectangular equations were very similar with and without the 0–100 coordinate constriction (see parenthesis in Table I), and that y_0 values of the crude equations were very close to 100. However, such resemblance was slightly lower for CPRG equations (for instance 61.26 versus 100 for y_0), what could represent an important variability in the sensing data for low analyte concentrations.

Allosteric Sensing Monitored by ONPG and CPRG

Since Galacton and FDG did not offered clear advantages over chromogenic substrates regarding sensitivity, signal-background ratio, range of response or reproducibility, we further tested CPRG and ONPG for a deeper comparative analysis. First, a pool of human, HIV-immune sera was used as analyte to explore the sensing behavior with natural samples. The activation profile is shown in Figure 2 and the relevant plot parameters at the bottom of Table I. Although the general behavior was slightly different than that observed with the model mAb (Fig. 1), the relative properties of both substrates indicated above were also conserved in the new context. In this regard, the saturation point (1/71.35) and the maximal activation factor (more than 700%) were both higher in CPRG than in ONPG (1/114.06) and 350%, respectively). In addition, CPRG hydrolysis permitted the detection of lower levels of antibodies in sera (1/38,998 vs. 1/8,369 with ONPG). In this case, the results were much less variable at low analyte concentrations than those observed with mAb, since the expected and actual y_0 values were very similar (Table I). The poor adjustment of activation values observed with both substrates at low sera dilutions, when the saturation point is reached, is irrelevant from a diagnostic point of view.

In a previous work and by using ONPG as substrate for NF795gpC (Ferraz et al., 2004), we observed that the ratio between the sensing signal and the background was largely dependent on the concentration of both enzyme and substrate in the reaction, defining time-evolving but consistent topographies. From the analysis of these maps, specific concentrations of the reaction components can be selected in which the activation factor is enhanced, a very convenient

Table I. Relevant parameters of NF795gp activation as monitored by diverse substrates.

		Equation parameters							X values for given activation values[b]	
Effector	Substrate	y_0[a]	a	SE a[c]	b	SE b	r^2	p	105% (ng/μL or dilution factor)	Inflexion (ng/μL or dilution factor)
Anti-P1 mAb	ONPG	100.00 (100.11)	161.79 (161.72)	3.10 (3.10)	0.75 (0.75)	0.06 (0.06)	0.995 (0.995)	<0.0001	0.024	1.543
	CPRG	100.00 (61.26)	484.73 (492.77)	52.10 (19.75)	6.79 (4.60)	2.31 (0.65)	0.966 (0.992)	<0.0001	0.071	5.198
	FDG	100.00 (107.25)	250.82 (262.04)	41.16 (50.20)	8.33 (10.85)	4.27 (5.85)	0.927 (0.930)	<0.0001	0.169	2.686
	Galacton	100.00 (98.73)	150.79 (151.78)	13.55 (13.43)	1.24 (1.21)	0.49 (0.47)	0.933 (0.933)	<0.0001	0.0425	1.109
Sera pool	ONPG	100.00 (104.74)	222.60 (218.97)	7.60 (7.40)	0.005 (0.005)	0.0009 (0.0009)	0.981 (0.978)	<0.0001	1/8369	1/114
	CPRG	100.00 (86.26)	648.46 (661.13)	19.63 (18.87)	0.003 (0.003)	0.0005 (0.0005)	0.984 (0.982)	<0.0001	1/38998	1/71

[a]Constants and r^2 values are given for equations adjusted to $y_0 = 100$, that are those used for further analysis. Parameters of equations obtained without this constriction are also given in parenthesis for comparison.
[b]X values are provided for 105% of NF795gpC activation and for the inflexion point in the activation curves plotted in Figure 1.
[c]Standard errors (SE) of the equation parameters.

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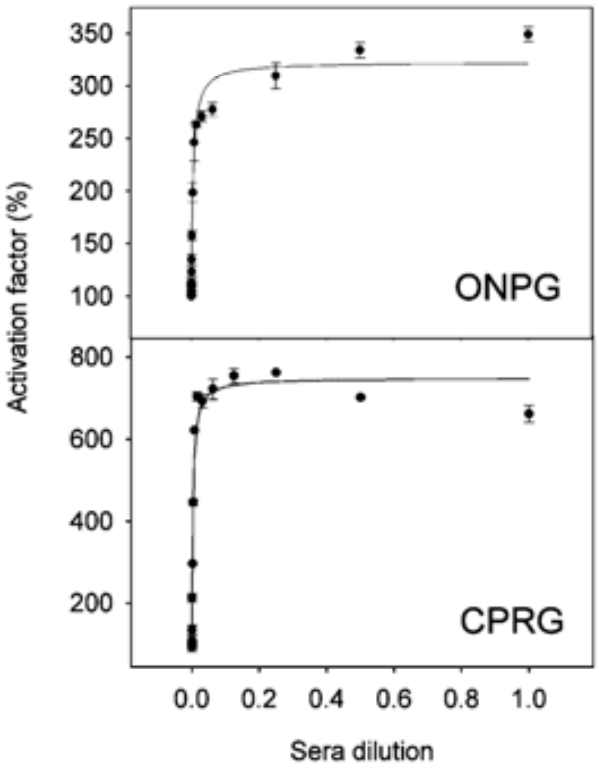

Figure 2. Enzymatic activation of NF795gpC upon exposure to an HIV-immune human sera pool as determined by the hydrolysis of ONPG or CPRG.

issue for diagnostic purposes. A comparative analysis of the sensing topography was then performed with both anti-P1 mAb and a human sera pool for CPRG and ONPG (Fig. 3). As observed with the effector mAb, the areas allowing amplified sensing signals were not coincident, being around 5–7 nM enzyme and 0.5–1.5 mM substrate for CPRG (panel C), and 7–11 nM enzyme and 2.5–4 mM substrate for ONPG (panel G). These areas were found more widely extended although the activation values were slightly lower when sensing antibodies in immune sera (panels D and H). In agreement with results shown in Figures 1 and 2, peak activation values were higher when using CPRG than ONPG (580.1% vs. 393.6% for the mAb and 426.3% and 300.1% for the immune sera). To evaluate the reproducibility of these results and the reliability of CPRG and ONPG as substrates for sensing, five different human HIV-immune sera were used as effectors to plot topological sensing maps with both substrates, under the concentration ranges of them and the enzyme indicated in Figure 3. Both the average sensing signal and the maximum activation values were obtained and compared by a correlation analysis. In Figure 4, regression lines are depicted showing a high consistence between data derived from both substrates (r^2 is 0.992 and 0.955 for average and maximal values, respectively, $P < 0.001$ in both cases), and again a higher sensing signal with CPRG, that for the most activating sample reached 1200% of the basal enzyme activity.

DISCUSSION

Allosteric enzymes respond to the binding of a specific ligand to a position distinct to the active site by a variation of their specific activity. This principle is obviously applicable to the specific detection of analytes of diagnostic interest. Being antibodies the most efficient allosteric effectors, infectious diseases are very appropriate for diagnosis through allosteric biosensing. Several β-galactosidase-based sensor prototypes have been previously developed that, by displaying immunodominant epitopes on their surface, are successfully activated by monoclonal antibodies but also by immune sera against either FMDV (Benito et al., 1996; Feliu et al., 2002) or HIV (Ferrer-Miralles et al., 2001). Antibody-mediated activation requires multiple and simultaneous monovalent contacts between this enzyme and the effector molecules (Alcalá et al., 2002) that modify the enzymatic constants presumably through alterations in the conformational architecture of the active site (Feliu et al., 1998, 2000). Since the modulation of substrate processing rate is the sensing signal itself (obtained by comparison of product formed in absence and in presence of the analyte), the specific substrate selected to determine the signal could be not irrelevant to the final sensing data. In this work, we have explored this issue by comparing different available substrates for this enzyme and that generate different kind of macroscopic signals. Interestingly, emission of light and fluorescence, although being themselves highly sensitive signals does not result into detection of lower analyte concentrations and both the sensitivity and activation profile are rather similar in all the tested substrates (Fig. 1, Table I). Contrarily, the chromogenic substrates ONPG and CPRG offer attractive properties such as lower detection limits and specially, in the case of CPRG, a wider activation range with a top signal/background ratio usually around 900% and up to 1200% (Figs. 1, 2, and 4). These high values can be approached by adjusting the concentrations of the reaction components, namely enzyme and substrate, to the single hot zone of the sensing reaction generated by CPRG (Fig. 3). Interestingly, although the substrate-dependence of the maximal activation value (parameter *a* in Table I) is not completely unexpected, the used substrate is also affecting the apparent dissociation constant of the effector antibody (namely parameter *b* in Table I). However, as the molecular size of substrates is different and the sensor is in fact activated by conformational modifications of the enzyme active site, each substrate could differently interpret, at the molecular level, the structural effects of antibody binding.

Sensitivity of allosteric β-galactosidase biosensors, being comparable to that automatized ELISA (Ferrer-Miralles et al., 2001), is not itself offering any additional advantaged for precise diagnosis under laboratory conditions. However, the simplicity and fastness of β-galactosidase activity detection through chromogenic components can make the sensing assay useful for high-throughput screening of a large number of samples in the field context, what could be carried out by non-skilled personnel through homogenous

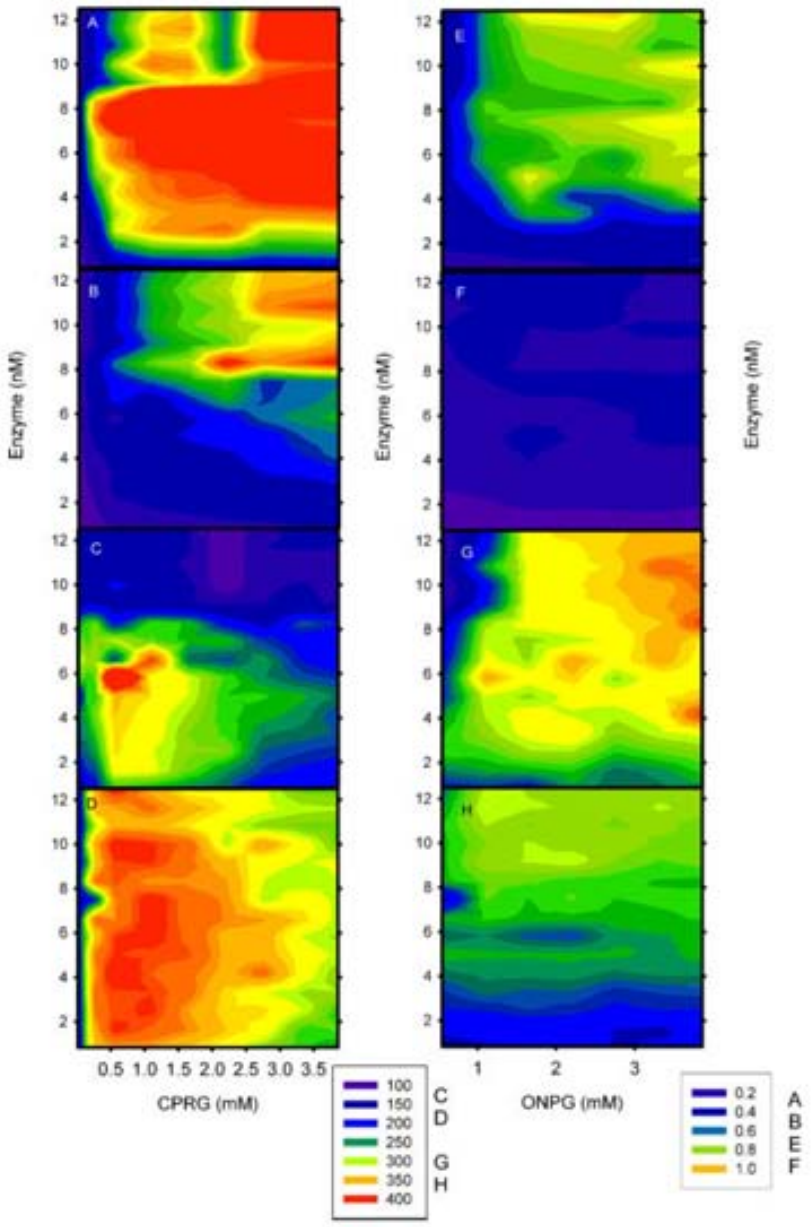

Figure 3. Topography of the sensing reaction promoted by the model anti-gp41 mAb (8.3 ng/μL, panels **A**, **B**, **C**, **E**, **F**, **G**) and measured by either CPRG (panels A, B, C) or ONPG (E, F, G), 20 min after the addition of the antibody. Direct absorbance values in presence of monoclonal antibody are depicted in panels A and E, background values in absence of monoclonal antibody in B and F, and the quotients (activation factors) in C and G. For comparison, the activation factors obtained by an HIV-immune human sera pool (dilution $\frac{1}{2}$) are also shown in panels **D** and **H** for CPRG and ONPG, respectively. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

microassays. This potential application approach is agreeably supported by the properties shown by CPRG but in particular by the enhanced signal/background ratios, higher than that offered by any other tested substrate and appropriate for screening under field conditions. On the other hand, the molecular mechanics supporting the highly divergent substrate production for CPRG hydrolysis in absence and in presence of the analyte deserve detailed investigation by molecular modeling.

Finally, the amperometric detection of specific antibodies in homogenous assays would also accomplish the requirements of a fast, field-framed diagnostic assay. However, the measure of the electric signal through glucose production resulting from lactose hydrolysis must largely overcome glucose levels as occurring in blood or sera. In our hands, glucose concentrations reached under the small-volume assay conditions appropriate with diagnosis have been too low for this purpose. Although β-galactosidase sensing by

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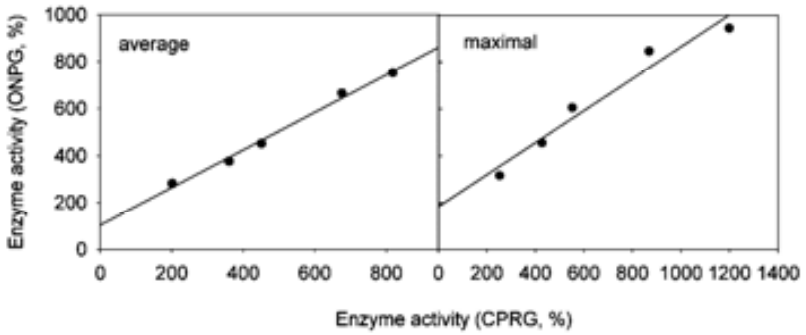

Figure 4. Correlation between average and higher activation factors from topography maps obtained by using either CPRG or ONPG, when analyzing five different, HIV-immune human sera.

electric determination would deserve further development for analytical purposes other than diagnosis, results presented here prompt the implementation and further investigation of CPRG for colorimetric sensing of specific antibodies.

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References

Alcalá P, Ferrer-Miralles N, Villaverde A. 2002. Co-activation of antibody-responsive, enzymatic sensors by a recombinant scFv antibody fragment produced in *E. coli*. Biotechnol Lett 24:1543–1551.

Benito A, Feliu JX, Villaverde A. 1996. Beta-galactosidase enzymatic activity as a molecular probe to detect specific antibodies. J Biol Chem 271(35):21251–21256.

Brennan CA, Christianson K, La Fleur MA, Mandecki W. 1995. A molecular sensor system based on genetically engineered alkaline phosphatase. Proc Natl Acad Sci USA 92(13):5783–5787.

Doi N, Yanagawa H. 1999. Design of generic biosensors based on green fluorescent proteins with allosteric sites by directed evolution. FEBS Lett 453(3):305–307.

Doi N, Yanagawa H. 2002. Evolutionary design of generic green fluorescent protein biosensors. Methods Mol Biol 183:49–55.

Feliu JX, Ramirez E, Villaverde A. 1998. Distinct mechanisms of antibody-mediated enzymatic reactivation in beta-galactosidase molecular sensors. FEBS Lett 438(3):267–271.

Feliu JX, Ramirez E, Villaverde A. 2000. Corrigendum to: Distinct mechanisms of antibody-mediated enzymatic reactivation in beta-galactosidase molecular sensors. FEBS Lett 473(1):123.

Feliu J. X, Ferrer-Miralles N, Blanco E, Cazorla D, Sobrino F, Villaverde A. 2002. Enhanced response to antibody binding in engineered beta-galactosidase enzymatic sensors. Biochim Biophys Acta 1596(2):212–224.

Ferguson A, Boomer RM, Kurz M, Keene SC, Diener JL, Keefe AD, Wilson C, Cload ST. 2004. A novel strategy for selection of allosteric ribozymes yields RiboReporter sensors for caffeine and aspartame. Nucleic Acids Res 32(5):1756–1766.

Ferraz RM, Aris A, Villaverde A. 2004. Profiling the allosteric response of an engineered beta-galactosidase to its effector, anti-HIV antibody. Biochem Biophys Res Commun 314(3):854–860.

Ferrer-Miralles N, Feliu JX, Villaverde A. 2000. Molecular mechanisms for antibody-mediated modulation of peptide-displaying enzyme sensors. Biochem Biophys Res Commun 275(2):360–364.

Ferrer-Miralles N, Feliu JX, Vandevuer S, Muller A, Cabrera-Crespo J, Ortmans I, Hoffmann F, Cazorla D, Rinas U, Prevost M, Villaverde A. 2001. Engineering regulable *Escherichia coli* beta-galactosidases as biosensors for anti-HIV antibody detection in human sera. J Biol Chem 276(43):40087–40095.

Legendre D, Soumillion P, Fastrez J. 1999. Engineering a regulatable enzyme for homogeneous immunoassays. Nat Biotechnol 17(1):67–72.

Miller JH. 1972. Experiments in molecular genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Saghatelian A, Guckian KM, Thayer DA, Ghadiri MR. 2003. DNA detection and signal amplification via an engineered allosteric enzyme. J Am Chem Soc 125(2):344–345.

Sambrook J, Fritsch E, Maniatis T. 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sekella PT, Rueda D, Walter NG. 2002. A biosensor for theophylline based on fluorescence detection of ligand-induced hammerhead ribozyme cleavage. RNA 8(10):1242–1252.

Ullmann A. 1984. One-step purification of hybrid proteins which have beta-galactosidase activity. Gene 29(1–2):27–31.

Villaverde A. 2003. Allosteric enzymes as biosensors for molecular diagnosis. FEBS Lett 554(1–2):169–172.

3.3. PUBLICATION III

HIGH-THROUGHPUT, FUNCTIONAL SCREENING OF THE ANTI-HIV-1 HUMORAL RESPONSE BY AN ENZYMATIC NANOSENSOR

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SUMMARY

Neutralizing antibodies are becoming of great interest in therapy and vaccine development to HIV. Several studies describe that these antibodies are able to promote conformational perturbations of the epitope and surrounding areas. In this work, the type of human antibodies acting as the most efficient sensor effector was investigated by exploring the relationship between antibody titre and the allosteric response. Different specific anti-HIV-1 gp41 antibodies present in sera samples (namely IgM, IgE, IgA, IgG1, IgG2, IgG3 and IgG4) were analyzed regarding their ability to activate the sensor, and in consequence, to conformationally modify the epitope. Sensor activation was significantly dependent on IgG4 antibody subpopulation binding, showing a specific ability of this type of antibodies in promoting conformational modifications in the target site.

The use of enzymatic nanosensors to high-throughput analysis of antibodies in sera was proposed.

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Short communication

High-throughput, functional screening of the anti-HIV-1 humoral response by an enzymatic nanosensor

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Abstract

The impact of antibodies on the target's epitope conformation is a major determinant of HIV-1 neutralization and a potential contributor to disease progression. We explore here a conformation-sensitive enzymatic nanosensor for the high-throughput functional screening of human anti-HIV-1 antibodies in sera. When displaying a model epitope from a gp41 immunodominant region (Env residues from 579 to 613), the sensing signal quantitatively distinguishes between adaptive and non-adaptive antibody binding. By using this tool, we have identified IgG4 as the immunoglobulin subpopulation most efficient in the structural modification of the target epitope.

Keywords: HIV; Antibody; IgG4; Immunosensor; Viral infection; Allosteric biosensor; Adaptive binding

1. Introduction

Antibody neutralization and viral escape have been regarded as important determinants in the dynamics of HIV-1 infection and progression to AIDS (Wei et al., 2003). Major immunodominant regions of gp41 and gp120 proteins contain most of the HIV-1 neutralizing epitopes identified so far (Broliden et al., 1992). In contrast to other viruses, many evasive mutations in HIV-1 map outside target neutralizing epitopes (Kwong et al., 2002; Watkins et al., 1996). This can be accounted by two intriguing escape mechanisms other than plain alterations of the antibody-binding site sequence. First, the occurrence of a gp41–gp120 glycan shield and its evolving structure, as supported by the modulation of the N-linked glycosilation pattern, allows an efficient carbohydrate masking of neutralizing epitopes (Wei et al., 2003; McCaffrey et al., 2004; Koch et al., 2003). Second, single amino acid substitutions inactivate remote epitopes by long distance structural alterations that are transmitted within, but also between gp41 and gp120 envelope proteins (Park et al., 1998, 2000; Zhang et al., 2002; Watkins et al., 1996; Thali et al., 1994). Such conformational camouflage indicates a high extent of flexibility of gp41–gp120 complexes, which allows protein domains to reorganize upon receptor binding (Myszka et al., 2000). In the context of this unusually high structural plasticity, neutralization of HIV-1 might be closely linked to the antibody-promoted conformational perturbation of the epitope itself and surrounding areas of influence. This possibility has been elegantly confirmed for the broadly neutralizing antibody 2F5, whose binding to gp41 precludes viral fusion through its conformational impact on gp41 (de Rosny et al., 2004). It has been suggested that other gp41-targeted neutralizing antibodies, namely 4E10 and Z13, could act in a similar way (de Rosny et al., 2004). Furthermore, the structural effect of anti-*env* antibodies was also observed with a set of 20 gp120-reactive monoclonal antibodies. The enthalpic and entropic changes of the former ligands correlated with their neutralization potency (Kwong et al., 2002). Interestingly, the ability to perform structural modifications is determined by the antibody and not by specific features of particular gp120 epitopes (Kwong et al., 2002).

However, the potency of human anti-HIV-1 sera to modify the target's structure remains to be analysed. Both X-ray anti-

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gen and antibody complex structure determination (Kwong et al., 1998) and thermodynamic measurement of antibody binding (Kwong et al., 2002) are not suitable for high-throughput screening. Moreover, determination of viral neutralization with homologous viruses is also not suitable for a large volume of samples and the biological significance of the neutralization of heterologous strains is at least controversial. Therefore, we propose here to use an enzymatic nanosensor, based on an engineered *Escherichia coli* β-galactosidase (NF795gpC), that can be useful to evaluate the structural impact of HIV-1 immune sera binding on a highly conserved antigenic segment of gp41 (Env residues from 579 to 613) (Viveros et al., 2000).

2. Material and methods

2.1. Protein, protein production and purification procedures

Protein NF795gpC is a modified *E. coli* β-galactosidase that displays four copies (one per subunit) of a 37-mer HIV-1 peptide, inserted into a solvent-exposed loop, close to the enzyme active sites. It includes the immunodominant B-cell epitope P1 from the gp41 structural protein, spanning amino acids 579–613 of the Env precursor (Ferrer-Miralles et al., 2001). The specific activity of the engineered enzyme is lower than that of the parental enzyme, but it is regulatable upon specific anti-HIV antibody binding. Protein NF795gpC was produced in *E. coli* BL26 as directed from the temperature sensitive expression vector pNF795gpC, and it was purified from crude cell extracts by affinity chromatography (Ullmann, 1984). The harvested protein was dialyzed against Z buffer and quantified by Bradford's procedure (Sambrook et al., 1989).

2.2. Sera and ELISA procedures

Both immune and non-immune human sera was obtained from indicated sources (Ferrer-Miralles et al., 2001) and also from the Hospital Universitari Germans Trias i Pujol, Badalona, Spain. An indirect, enzyme linked immunoassay (ELISA) was performed by using saturating amounts of NF795gpC (7 pmol per well) as antigen. Standard procedures were applied as described elsewhere (Ferrer-Miralles et al., 2001). All sera were diluted at 1/500 and 100 μl of such solutions were added to each well. Different types of second anti-human antibodies were used at optimal dilutions (anti-IgG, 1/2000 from Pierce; anti-IgG1, 1/500; anti-IgG2, 1/2000; anti-IgG3, 1/1000; anti-IgG4, 1/2000; anti-IgM, 1/2000; anti-IgA, 1/1000 and anti-IgE, 1/500 from Southern Biotechnology Associates, Inc.)

For the comparison between monoclonal antibodies F240 and anti-P1, we used several antibody concentrations up to 50 ng/μl, and 1/2000 diluted anti-human or anti-mouse (Bio-Rad Laboratories) secondary antibodies. The reaction was developed by using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) from Sigma, according to the instructions given by the manufacturer. Samples were analysed in triplicate.

2.3. β-Galactosidase activity assay

Activation assays were performed in ELISA microplates as described before (Ferraz et al., 2004). Briefly, monoclonal antibodies F240 and anti-P1 were diluted in buffer Z with 0.65 pmol of NF795gpC and further incubated for 60 min at 25 °C in a total volume of 80 μl. Otherwise, sera diluted at 1/4 were mixed with 0.5 pmol of enzyme and incubated for 60 min at 25 °C. Then, 40 μl of *ortho*-nitrophenyl β-D-galactopyranoside (ONPG) were used to determine mAb-mediated sensor activation and 40 μl of chlorophenol red β-D-galactopyranoside (CPRG) in samples activated with sera, both substrates in a final concentration of 0.33 mg/ml. After 30 min of reaction, absorbance was determined at 414 and 540 nm, respectively, with a microtitre plate Labsystems IEMS reader. The allosteric signal was measured as the percentage of product hydrolysed by the β-galactosidase sensor in presence of effector, relative to that hydrolysed in absence of any analyte, through absorbance quotients (therefore activation values are given as 100% or higher) (Benito et al., 1996). All the assays were performed in triplicate.

2.4. Data analysis

The allosteric signals obtained with sera panels as described above were straightforward used for correlation with different parameters, namely ELISA absorbances for isotype determination, AxSYM rough data as indicated (Ferrer-Miralles et al., 2001), $CD4^+$ or $CD8^+$ cell concentration and HIV-1 RNA concentration. Correlation analysis were done by using either activation data obtained under the optimal enzyme and substrate concentrations for a higher signal:background ratio (4.16 nM NF795gpC and 1.66 mM CPRG; (Ferraz et al., 2004) or data sets collected at different enzyme and substrate concentrations namely 2.08, 4.16, 6.26, 8.33 and 10.41 nM NF795gpC, and 1.66, 3.32, 6.60 and 10.00 mM CPRG. The significance of both type of correlation was similar and they are shown in Table 3.

2.5. Protein–antibody interaction analysis

Biacore analysis was performed by using a BIAcore1000 instrument, BIAcore1000 control software version 2.3 and BIAevaluation Software v. 4.1 (Biacore AB, Uppsala, Sweden). Each monoclonal antibody was covalently attached to a carboximetilated dextran matrix of a Sensor Chip CM5 Research grade (Biacore AB, Uppsala, Sweden) using amine coupling chemistry as described by the manufacturer. Briefly, the carboxyl groups of the chip surface were activated by exposing to a mixture of 200 mM *N*-ethyl-*N*′-dimethylaminopropyl carbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) for 7 min. The two monoclonal antibodies F240 and anti-P1 were immobilised into two different flow cells at 25 μg/ml in HEPES-buffered saline (HBS, 10 mM HEPES, 150 mM NaCl, 0.05% Tween 20 and 3 mM EDTA at pH 3.45 and 3.85, respectively), followed by injection of 1 M ethanolamine to block the unreacted groups. Protein NF795gpC was used as analyte. Binding assays were performed at 25 °C in HBS pH 7.4 at a flow rate of 3 μl/min, by adding 70 μl of protein solution. Between each cycle of analysis,

5 μl of 100 mM NaOH were injected at a flow rate of 10 μl/min for the regeneration of the ligand. Data from every flow cell exposed to different amounts of NF795gpC were analysed to obtain the values of association and dissociation rate constants and the affinity constant KD (dissociation constant/association constant). Association and dissociation rate constants were calculated separately by 1:1 (Langmuir) model, available in the BIAevaluation Software v. 4.1 (Biacore AB, Uppsala, Sweden).

3. Results

The viral peptide displayed by NF795gpC contains a major neutralizing, disulphide bridge-free B-cell epitope (named P1 and spanning residues from 589 to 603) displayed in its helical, native conformation (Ferrer-Miralles et al., 2001). The modified β-galactosidase (NF795gpC) enzyme displays a total of four copies (one per monomer) of the viral peptide on solvent-exposed surfaces and responds allosterically to antibody ligands by an up-shift in its specific activity (Ferrer-Miralles et al., 2001; Ferraz et al., 2004). The conformational activation of NF795gpC does not involve alterations of the quaternary structure and it is irrespective of antibody multivalency (Ferrer-Miralles et al., 2001).

As shown in Table 1, the activation of NF795gpC was linearly dependent on the effector concentration, either polyclonal sera or monoclonal antibodies. In addition, the extent at which this sensor is activated by human (immune plus non-immune) sera showed a significant and very high linear correlation with anti-HIV-1 antibody titers, as demonstrated by both the automated AxSYM immunoassay and a manual ELISA (Table 1). However, when the analysis was limited to immune sera no dependence was observed. Importantly, this result indicated that the mere interaction with the antibody did not necessarily result

Table 1
Correlation parameters between enzymatic activation and antibody binding measured by two alternative procedures

	AxSYM[a]	ELISA[b]
Immune and non-immune sera	$r = 0.793$, $p < \mathbf{0.0001}$, $n = 61$	$r = 0.672$, $p < \mathbf{0.0001}$, $n = 46$
Immune sera[c]	$r = 0.296$, $p = 0.098$, $n = 32$	$r = 0.027$, $p = 0.890$, $n = 29$

[a] Anti-HIV antibody titer in 61 infected and non-infected individuals were obtained by AxSYM in a previous study (Ferrer-Miralles et al., 2001). Detailed procedures for the sensing reaction (lasting 30 min) are explained in detail elsewhere (Ferraz et al., 2004). The linearity of the sensing response to the effector antibody concentration has been assessed for NF795gpC with the anti-P1 monoclonal antibody (Ferrer-Miralles et al., 2001) ($p < 0.001$), and with a pool of sera from 31 HIV-1-infected individuals ($p < 0.001$). Such linear response is in agreement with that found with related β-galactosidase nanosensors displaying different antigens (Benito et al., 1996).

[b] Indirect ELISA was performed manually in microtiter plates by using an anti-IgG secondary antibody.

[c] Possible relationship between ELISA and enzymatic activation data other than linear dependence were also explored, in all cases rendering non-significant correlation factors ($p > 0.10$, not shown).

Table 2
Binding and activation of NF795gpC with two monoclonal antibodies directed against epitope P1

	Monoclonal antibody and reference		
	F240 (Cavacini et al., 1998)	Anti-P1 (Ferrer-Miralles et al., 2001)	p^a
Sensor activation (%)[b]	201.87 ± 15.31	252.05 ± 4.02	**0.046**
ELISA (% of maximal signal)[c]	99.66 ± 0.18	87.124 ± 14.93	0.142
KD (M)[c]	$2.72 \times 10^{-8} \pm 2.13 \times 10^{-8}$	$2.93 \times 10^{-8} \pm 9.28 \times 10^{-9}$	1.000

[a] The significance of the different values obtained for the two tested antibodies was analysed by a one-factor ANOVA test.

[b] Sensor activation was determined at 33.0 ng/μl antibody and 5.4 nM NF795gpC.

[c] Both binding and dissociation constants were measured by using NF795gpC as antigen. BIACORE experiments were performed according to common procedures. All the experiments were performed at least in triplicate.

Table 3
Correlation parameters (namely r and p) for enzymatic sensing and clinical features in HIV-1-infected individuals ($n = 15$)

		Antibody titter								Clinical parameter		
		IgA	IgM	IgE	IgG	IgG1	IgG2	IgG3	IgG4	VL	$CD4^+$	$CD8^+$
Discrete signal[a]	r[c]	0.184	0.275	0.047	0.028	0.379	0.008	0.199	0.579	0.006	0.090	0.271
	p	0.512	0.442	0.866	0.921	0.164	0.978	0.496	**0.023**	0.974	0.642	0.171
Average signal[b]	r	0.020	0.072	0.043	0.065	0.369	0.055	0.224	0.580	0.051	0.079	0.270
	p	0.465	0.814	0.878	0.817	0.176	0.846	0.423	**0.023**	0.791	0.683	0.173

[a] The sensing signal was determined as the quotient between the product of CPRG hydrolysis mediated by NF795gpC, formed in presence and absence of sera. The sensing reaction was performed at 4.16 nM NF795gpC and 1.66mM CPRG, conditions in which the background signal has been found minimal (Ferraz et al., 2004). All the reactions were performed in triplicate.

[b] Correlations were also explored by using an average sensing signal obtained under different enzymatic reaction conditions. In particular, sera were tested for sensor activation in all possible combinations of 2.08, 4.16, 6.26, 8.33 and 10.41 nM NF795gpC, and 1.66, 3.32, 6.60 and 10.00 mM CPRG. Since all the reactions were performed in triplicate, the average sensing signal for each specific serum was obtained from about 50 independent enzymatic tests. p-Values lower than 0.05 are indicated in boldface.

[c] In the correlation analysis, ELISA absorbance units (A_{405}) was considered for antibodies, HIV-1 RNA copies per millilitres for viral load, and number of cells per microlitres for $CD4^+$ and $CD8^+$.

in important perturbations of the target epitope. Moreover, reactive antibodies may be differently competent (as expected) in epitope modification. This possibility was confirmed by the divergent behaviour of two selected anti-gp41 monoclonal antibodies directed against the immunodominant epitope displayed on NF795gpC. These antibodies exhibited indistinguishable affinity constants to the viral peptide, but activated the sensor with significantly different efficiencies (Table 2).

To identify functional antibody populations elicited during the immune response to viral infection we compared sensor activation levels with the occurrence of anti-gp41 IgA, IgE, IgM and IgG, as well as with the different IgG isotypes, in immune sera, from a group of patients ($n = 15$) with different viral loads, $CD4^+$ and $CD8^+$ titers. As expected, β-galactosidase activation did not correlate with those clinical parameters but neither with binding of IgA, IgM, IgE, total IgG and IgG1, IgG2 and IgG3 isotypes (Table 3). Interestingly, sensor activation was instead significantly dependent on IgG4 binding ($p = 0.023$; $n = 15$). To confirm such correlation, we determined the occurrence of anti-HIV IgG4 in a larger number of sera samples to be added in our analysis (not shown), and the anti-HIV IgG4 dependence of the sensor activation became then much more obvious ($n = 55$; $p = 0.001$).

4. Discussion

Allosteric biosensors are useful tools for the diagnosis of infectious diseases and other analytical purposes (Villaverde, 2003). By exploring solvent-exposed sites of the *E. coli* β-galactosidase, permissive to peptide insertions (Benito et al., 1995; Feliu and Villaverde, 1998), we have constructed sets of hybrid enzymes containing different viral epitopes that are activated by anti-peptide antibodies (Benito et al., 1996; Feliu et al., 1998b; Ferrer-Miralles et al., 2000) and useful for the detection of the infection of either foot-and-mouth disease virus (Feliu et al., 2002) or HIV-1 (Ferrer-Miralles et al., 2001). Interestingly, structurally different foreign peptides as those used from foot-and-mouth disease virus VP1 capsid protein (Feliu et al., 1998a) and HIV-1 gp41 protein (Ferrer-Miralles et al., 2001) are efficient as receptors for allosteric biosensing through antibody-dependent induced fit, although only defined acceptor sites in the enzyme efficiently support antibody-mediated activation (Feliu et al., 1998b; Ferrer-Miralles et al., 2001). One of such sites, namely between enzyme residues 795 and 796, has been used to construct the model sensor NF795gpC, whose efficiency as a diagnostic tool is comparable to that of conventional automated immunoassays (Ferrer-Miralles et al., 2001). By using this sensor, displaying an immunodominant epitope from HIV-1 gp41, we have proved here that the presence of anti-peptide IgG4 antibodies are main contributors of sensor activation, what indicates a specific ability of such antibody population in promoting conformational perturbations in the target antigenic site.

The occurrence of IgG isotypes in the plasma of infected individuals is becoming a sudden matter of exciting attention, as they might become useful markers for AIDS progression (Martinez et al., 2005; Abbas et al., 2005). In this regard, the occurrence of IgG4 in slow progressors correlates with $CD8^+$ cell count, while in rapid progressors, IgG4 correlates with the percentage of $CD4^+$ cells (Abbas et al., 2005). Although these data are still partial and IgG4 has been sometimes used in combination with other parameters for correlation analysis (Martinez et al., 2005), these preliminary studies indicate that IgG4 might play a functional role in controlling HIV-1 replication. On the other hand, the data presented in this work indicate that this antibody subpopulation may exhibit an unusual ability of epitope modification, that could be eventually related to particular neutralizing abilities. The unusual structural properties of IgG4 (namely a short bridge and the lack of covalent interactions between heavy chains among others) (Aalberse and Schuurman, 2002), could contribute to its particular impact on the target epitope as revealed here. The possible influence of this fact on the progression of the viral infection would require further and deep investigation.

The results presented here prompt the consideration of enzymatic nanosensors for the high-throughput analysis of antibodies present in sera samples. In the case of HIV-1, the conformational perturbation of a major gp41 epitope by the antibody adaptive binding seems to be of particular biological relevance, and it is predominantly mediated by the IgG4 population.

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References

Aalberse, R.C., Schuurman, J., 2002. IgG4 breaking the rules. Immunology 105, 9–19.

Abbas, A., Vasilescu, A., Do, H., Hendel, H., Maachi, M., Goutalier, F.X., Regulier, E.G., Rappaport, J., Matsuda, F., Therwath, A., Aucouturier, P., Zagury, JF., 2005. Analysis of IGG and IGG4 in HIV-1 seropositive patients and correlation with biological and genetic markers. Biomed. Pharmacother. 59, 38–46.

Benito, A., Feliu, J.X., Villaverde, A., 1996. Beta-galactosidase enzymatic activity as a molecular probe to detect specific antibodies. J. Biol. Chem. 271, 21251–21256.

Benito, A., Mateu, M.G., Villaverde A, 1995. Improved mimicry of a foot-and-mouth disease virus antigenic site by a viral peptide displayed on beta-galactosidase surface. Biotechnology (N.Y.) 13, 801–804.

Broliden, P.A., von Gegerfelt, A., Clapham, P., Rosen, J., Fenyo, E.M., Wahren, B., Broliden, K., 1992. Identification of human neutralization-inducing regions of the human immunodeficiency virus type 1 envelope glycoproteins. Proc. Natl. Acad. Sci. U.S.A. 89, 461–465.

Cavacini, L.A., Emes, C.L., Wisnewski, A.V., Power, J., Lewis, G., Montefiori, D., Posner, MR., 1998. Functional and molecular characterization of human monoclonal antibody reactive with the immunodominant region of HIV type 1 glycoprotein 41. AIDS Res. Hum. Retroviruses 14, 1271–1280.

de Rosny, E., Vassell, R., Jiang, S., Kunert, R., Weiss, CD., 2004. Binding of the 2F5 monoclonal antibody to native and fusion-intermediate forms

of human immunodeficiency virus type 1 gp41: implications for fusion-inducing conformational changes. J. Virol. 78, 2627–2631.

Feliu, J.X., Benito, A., Oliva, B., Aviles, F.X., Villaverde, A., 1998a. Conformational flexibility in a highly mobile protein loop of foot-and-mouth disease virus: distinct structural requirements for integrin and antibody binding. J. Mol. Biol. 283, 331–338.

Feliu, J.X., Ferrer-Miralles, N., Blanco, E., Cazorla, D., Sobrino, F., Villaverde, A., 2002. Enhanced response to antibody binding in engineered beta-galactosidase enzymatic sensors. Biochim. Biophys. Acta 1596, 212–224.

Feliu, J.X., Ramirez, E., Villaverde, A., 1998b. Distinct mechanisms of antibody-mediated enzymatic reactivation in beta-galactosidase molecular sensors. FEBS Lett. 438, 267–271.

Feliu, J.X., Villaverde, A., 1998. Engineering of solvent-exposed loops in *Escherichia coli* beta-galactosidase. FEBS Lett. 434, 23–27.

Ferraz, R.M., Aris, A., Villaverde, A., 2004. Profiling the allosteric response of an engineered beta-galactosidase to its effector, anti-HIV antibody. Biochem. Biophys. Res. Commun. 314, 854–860.

Ferrer-Miralles, N., Feliu, J.X., Vandevuer, S., Muller, A., Cabrera-Crespo, J., Ortmans, I., Hoffmann, F., Cazorla, D., Rinas, U., Prevost, M., Villaverde, A., 2001. Engineering regulable *Escherichia coli* beta-galactosidases as biosensors for anti-HIV antibody detection in human sera. J. Biol. Chem. 276, 40087–40095.

Ferrer-Miralles, N., Feliu, J.X., Villaverde, A., 2000. Molecular mechanisms for antibody-mediated modulation of peptide-displaying enzyme sensors. Biochem. Biophys. Res. Commun. 275, 360–364.

Koch, M., Pancera, M., Kwong, P.D., Kolchinsky, P., Grundner, C., Wang, L., Hendrickson, W.A., Sodroski, J., Wyatt, R., 2003. Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition. Virology 313, 387–400.

Kwong, P.D., Doyle, M.L., Casper, D.J., Cicala, C., Leavitt, S.A., Majeed, S., Steenbeke, T.D., Venturi, M., Chaiken, I., Fung, M., Katinger, H., Parren, P.W., Robinson, J., Van Ryk, D., Wang, L., Burton, D.R., Freire, E., Wyatt, R., Sodroski, J., Hendrickson, W.A., Arthos, J., 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature 420, 678–682.

Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J., Hendrickson, WA., 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 393, 648–659.

Martinez, V., Costagliola, D., Bonduelle, O., N'go, N., Schnuriger, A., Theodorou, I., Clauvel, J.P., Sicard, D., Agut, H., Debre, P., Rouzioux, C., Autran, B., 2005. Combination of HIV-1-specific CD4 Th1 cell responses and IgG2 antibodies is the best predictor for persistence of long-term non-progression. J. Infect. Dis. 191, 2053–2063.

McCaffrey, R.A., Saunders, C., Hensel, M., Stamatatos, L., 2004. N-linked glycosylation of the V3 loop and the immunologically silent face of gp120 protects human immunodeficiency virus type 1 SF162 from neutralization by anti-gp120 and anti-gp41 antibodies. J. Virol. 78, 3279–3295.

Myszka, D.G., Sweet, R.W., Hensley, P., Brigham-Burke, M., Kwong, P.D., Hendrickson, W.A., Wyatt, R., Sodroski, J., Doyle, ML., 2000. Energetics of the HIV gp120-CD4 binding reaction. Proc. Natl. Acad. Sci. U.S.A. 97, 9026–9031.

Park, E.J., Gorny, M.K., Zolla-Pazner, S., Quinnan Jr., G.V., 2000. A global neutralization resistance phenotype of human immunodeficiency virus type 1 is determined by distinct mechanisms mediating enhanced infectivity and conformational change of the envelope complex. J. Virol. 74, 4183–4191.

Park, E.J., Vujcic, L.K., Anand, R., Theodore, T.S., Quinnan Jr., G.V., 1998. Mutations in both gp120 and gp41 are responsible for the broad neutralization resistance of variant human immunodeficiency virus type 1 MN to antibodies directed at V3 and non-V3 epitopes. J. Virol. 72, 7099–7107.

Sambrook, J., Fritsch, E., Maniatis, T., 1989. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Thali, M., Charles, M., Furman, C., Cavacini, L., Posner, M., Robinson, J., Sodroski, J., 1994. Resistance to neutralization by broadly reactive antibodies to the human immunodeficiency virus type 1 gp120 glycoprotein conferred by a gp41 amino acid change. J. Virol. 68, 674–680.

Ullmann, A., 1984. One-step purification of hybrid proteins which have beta-galactosidase activity. Gene 29, 27–31.

Villaverde, A., 2003. Allosteric enzymes as biosensors for molecular diagnosis. FEBS Lett. 554, 169–172.

Viveros, M., Dickey, C., Cotropia, J.P., Gevorkian, G., Larralde, C., Broliden, K., Levi, M., Burgess, A., Cao, C., Weiner, D.B., Agadjanyan, M.G., Ugen, KE., 2000. Characterization of a novel human immunodeficiency virus type 1 neutralizable epitope within the immunodominant region of gp41. Virology 270, 135–145.

Watkins, B.A., Buge, S., Aldrich, K., Davis, A.E., Robinson, J., Reitz Jr., M.S., Robert-Guroff, M., 1996. Resistance of human immunodeficiency virus type 1 to neutralization by natural antisera occurs through single amino acid substitutions that cause changes in antibody binding at multiple sites. J. Virol. 70, 8431–8437.

Wei, X., Decker, J.M., Wang, S., Hui, H., Kappes, J.C., Wu, X., Salazar-Gonzalez, J.F., Salazar, M.G., Kilby, J.M., Saag, M.S., Komarova, N.L., Nowak, M.A., Hahn, B.H., Kwong, P.D., Shaw, GM., 2003. Antibody neutralization and escape by HIV-1. Nature 422, 307–312.

Zhang, P.F., Bouma, P., Park, E.J., Margolick, J.B., Robinson, J.E., Zolla-Pazner, S., Flora, M.N., Quinnan Jr., G.V., 2002. A variable region 3 (V3) mutation determines a global neutralization phenotype and CD4-independent infectivity of a human immunodeficiency virus type 1 envelope associated with a broadly cross-reactive, primary virus-neutralizing antibody response. J. Virol. 76, 644–655.

3.4. PUBLICATION IV

ALLOSTERIC MOLECULAR SENSING OF ANTI-HIV ANTIBODIES BY AN IMMOBILIZED ENGINEERED β-GALACTOSIDASE

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SUMMARY

Activation properties of the protein NF795gpC bound to a surface such as agarose were analyzed. This is an essential step in order to develop new and portable versions of the sensor, for the future implementation of optimized devices able to detect HIV infection in an easy and reliable way and by non-skilled personnel.

The protein NF795gpC was immobilized into an agarose support. Anti-HIV antibodies were analyzed in this situation and the sensing signals compared with the results obtained in the common assay. Higher activation values were obtained with the free enzyme, but the immobilized version exposed to HIV-infected patients sera, showed a sufficient increase of activity for sensing purposes. Furthermore, it was demonstrated that biosensor is stable enough to perform measurements at environment temperature.

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Allosteric molecular sensing of anti-HIV antibodies by an immobilized engineered β-galactosidase

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Abstract

The main objective is to demonstrate the feasibility of qualitative and semi-quantitative determination of anti-HIV antibodies, based on the allosteric response of an immobilized engineered *Escherichia coli* β-galactosidase. The suitability of this enzyme and the conditions for homogeneous assay had been previously demonstrated. In the present work, the operational conditions for engineered *E. coli* β-galactosidase immobilization on activated agarose supports have been determined. A solid biocatalyst has been prepared and characterized in order to determine the activation degree of the immobilized enzyme in the presence of anti-HIV antibodies. The solid-phase biocatalyst has been shown to be able to modulate its activity and produce an unequivocal signal in presence of anti-HIV containing sera from different patients. Finally, the conceptual design of a possible assay based on the above methodology is proposed.

Keywords: Immobilized engineered β-galactosidase; Anti-HIV antibodies sensing; Allosteric enzymes

1. Introduction

Biosensors are hybrid devices that convert chemical information into an analytical signal by using a biochemical mechanism [1]. Among protein biosensors, those based on the allosteric responses of modified enzymes [2] are promising instruments for fast and ultra-fast molecular diagnosis through specific antibody detection [3]. In them, the analyte antibody interacts specifically with the receptor, a foreign antigenic peptide displayed in the vicinity of the enzyme active site. As a result of such interaction and by means of the induced fit, the functional architecture of the active site is modified and the catalytic activity modulated in a detectable way. The enzyme itself acts then as a physicochemical transducer that amplifies the signal resulting from such interaction to macroscopical level. A rather short list of natural enzymes have been engineered as allosteric biosensors including β-galactosidase, alkaline phosphatase and TEM-1 β-lactamase [2], being all of them suitable for homogeneous immunoassays. In particular, *Escherichia coli* β-galactosidase has been successfully tailored for the diagnosis of either foot-and-mouth disease [4,5] and human immunodeficiency virus (HIV) infection [6,7], and conditions for homogenous assays in liquid phase completely established [8].

For many diagnostic applications, in both human and animal health, it would be desirable the development of a solid-phase biosensor version that could be used by non-skilled personnel in a simple and reliable way. This is especially significant in field campaigns and when a large number of samples are to be screened. For anti-HIV antibodies detection, the current assays are different immunodetection methods [9,10], and there is a great interest in field and fast optimized methods [11,12]. In this context, chromogenic substrates would be also very appropriate for the qualitative and semi-quantitative identification of antibodies in immune sera or any other relevant

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analytes. Among the different substrates available for the enzyme, those rendering colored products, namely *ortho*-nitrophenyl β-D-galactopyranoside (ONPG) and chlorophenol red β-D-galactopyranoside (CPRG), have been observed as the most appropriate for antibody biosensing, because of the low detection limits and the high signal-background ratio deriving from their use [13].

An initial but critical step in this direction would be the immobilization of the engineered biocatalyst in a stable and functional way, still not reported by any allosteric enzyme. In this work, using a modified *E. coli* β-galactosidase responsive to anti-HIV antibodies, we prove that the immobilization in glyoxal-agarose supports results in enzymes fully responsive to immune human sera. The objective is to demonstrate the usefulness of the immobilized allosteric enzyme for qualitative and semi-quantitative detection of HIV antibodies, opening possible future biosensor developments.

2. Materials and methods

2.1. Proteins, protein expression and sera

The proteins used were either commercial β-galactosidase from *E. coli* (Roche Diagnostics S.L.) or NF795gpC, a modified *E. coli* β-galactosidase which contains an insertion of the epitope P1 from the protein gp41 of HIV-1, between residues 795 and 796 of every enzyme subunit [7]. The viral epitope spans the amino acids from 579 to 613 of the Env precursor and is a major immunogenic HIV-1 β-cell epitope. The peptide is accommodated into a solvent-exposed loop near the enzyme active site and fully available for interaction with specific antibodies. While the mutant protein displays a catalytic activity lower than the parental version, the presence of a specific anti-epitope antibody enhances this activity in a dose-dependent manner [4]. Protein NF795gpC was produced in *E. coli* BL26 using the temperature sensitive plasmid pNF795gpC [7] and it was purified by affinity chromatography [14] using buffer Z in the dialysis procedures [15].

For biosensing experiments we used either a pool of 36 human sera from HIV-1-infected individuals or some of them individually. The sera samples were generously provided by Annette Müller (Microcoat) [7].

2.2. Immobilization support and activation procedure

Agarose beads 10BCL and 4BCL from Hispanagar were used as supports and their activation process is described elsewhere [16]. Briefly, after the etherification of agarose with glycidol (2,3-epoxypropanol, from Sigma) during 19 h at 20 °C, it was oxidized using $NaIO_4$ 0.1 M (Sigma) and the amount of aldehyde groups formed after 35 min of incubation was measured through an enabled reaction using IK 10% and saturated $NaHCO_3$. The number of aldehyde groups available to link the ligand was calculated by analyzing the consumed periodate [16].

2.3. Protein immobilization into agarose and stability analysis

The immobilization process was carried out at pH 10.1 for the commercial β-galactosidase (Roche Diagnostics S.L.) and at pH 9.5 for NF795gpC, at 4 °C, under gentle stirring. A solution of 100 mM $NaHCO_3$ at the operation pH was mixed with the enzymatic solution leading to a final concentration of 50 mM $NaHCO_3$. An appropriate volume of this solution was mixed with activated agarose, offering approximately 30 AU/mL of agarose support. The remaining volume was used as a reference (blank) to test soluble enzyme stability under immobilization conditions. The catalytic activity of the blank, supernatant and total suspension was measured at different times withdrawing samples of 20 or 40 μL as described before [17]. The reduction of the Schiff bases was performed employing a $NaBH_4$ concentration of 1 mg/mL during 30 min [18]. Finally, the enzyme derivative was separated from the supernatant by filtration, washed with water and stored at 4 °C.

2.4. Enzymatic assay

Twenty microliters of either protein solution or immobilization solution (supernatant or blank) were mixed with 335 μL of 6 mM ONPG from Sigma and 645 μL of Z buffer pH 8.4. Activity was measured using a spectrophotometer Cary Varian 2, following the increase in absorbance at 420 nm in 1 mL-cuvettes. For the immobilized NF795gpC, the assay was done using 40 μL of derivative agarose suspension with a gentle stirring in a 2 mL cuvette. An activity unit (AU) was defined as the amount of β-galactosidase which hydrolyses 1 μmol ONPG/min at 28 °C and pH 8.4, using a molar extinction coefficient $\varepsilon = 4155\ M^{-1}\ cm^{-1}$.

2.5. Activation assays

Three hundred microliters of either the sera pool or a selected single serum was used for the activation assays. Relative enzymatic activities were obtained by comparing the activity values (AU/mL) from the samples in presence of immune sera related to those ones obtained in absence of anti-HIV antibodies. Thus, we consider a 100% activity value as that obtained in absence of antibody. From replicated experiments, a mean deviation of ±10% was calculated.

3. Results and discussion

The purpose of the present work was to explore the ability of an engineered β-galactosidase enzyme (protein NF795gpC) to allosterically perform HIV antibody determination upon immobilization in a solid phase. This is a first and necessary step for the further development of a portable device to be used under field conditions by non-skilled personnel. This assay kit would represent an advantageous method of screening of HIV-1 infection in situations in which sophisticated laboratory resources are absent.

The functioning of such device implies the availability of a solid-phase stable biocatalyst acting as sensor and, as the enzymatic activity is modulated by the presence of the analyte (anti-HIV antibodies), an unequivocal signal (optical in this case) has to be obtained.

3.1. Definition of immobilization procedure

Preliminary selection of the appropriate conditions for immobilization has been performed using a commercial β-galactosidase. Glyoxal-agarose was chosen as support because of its well-known good performance with a great number of enzymes [19]. Enzymes can be immobilized by multipoint covalent attachment to the generated aldehyde residues on the support through their amine groups. The employed procedure is detailed in Section 2, including the steps of support activation, enzyme immobilization and sodium borohydride reduction to transform the Schiff's base to a stable single C–N bond and the remaining free aldehyde residues to non-reactive hydroxyl groups [17].

The immobilization of native β-galactosidase was assayed employing 4BCL and 10BCL agarose supports with the maximum activation degree and at alkaline pH values to ensure a high number of non-protonated amine residues in the protein surface (results not shown). The best immobilization results were

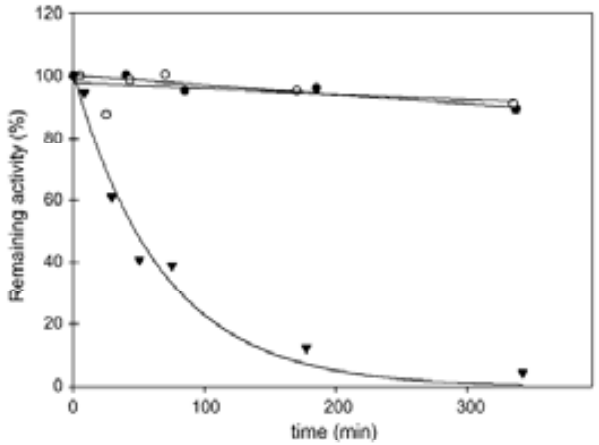

Fig. 1. Time course of the immobilization process of a commercial β-galactosidase in 4BCL agarose. (●) Activity evolution of the blank (β-galactosidase without support). (○) Suspension activity, accounting for the enzymatic activity in the supernatant plus the one immobilized onto agarose. (▼) Activity of the remaining protein in the supernatant.

obtained with 4BCL agarose as immobilization support and the time evolution of the immobilization process is shown in Fig. 1.

Along time, we observed an accentuated decrease on the supernatant activity but in the suspension and the blank it remained stable, indicating that protein progressively linked to agarose reactive aldehyde groups.

The immobilization yield was around 100%, calculated as initial offered activity (100%) minus percentage of initial activity in the supernatant. The retained activity in the derivative was calculated by subtracting the remaining supernatant β-galactosidase activity from the suspension activity (in % of initial activity). Using data from Fig. 1, 85% of the offered activity was recovered in the immobilized derivative.

3.2. Immobilization of engineered β-galactosidase

The allosteric engineered *E. coli* β-galactosidase NF795gpC has been prepared by insertion of a peptide that reproduces the immunodominant β-cell epitope P1 from the gp41 structural protein. This enzyme exhibits lower hydrolysis rate of the substrate ONPG than the native one but the presence of antibodies directed to this peptide significantly increases the enzymatic activity [6–8]. The engineered β-galactosidase enzyme has been produced in *E. coli* according to the procedure explained in Section 2.

To translate the immobilization methodology from the commercial to the recombinant target enzyme, its stability under the alkaline conditions required for immobilization was studied and the results are presented in Fig. 2. The engineered β-galactosidase is rather unstable at pH 10.1, but at pH 9.5 its stability is good enough for immobilization purposes (Fig. 2). This pH was employed in subsequent immobilization runs, as a condition leading to a reasonable number of non-protonated residues and maintaining stability, in spite of lower immobilization rate than at pH 10.1. A representative immobilization time-course result is shown in Fig. 3. As observed, NF795gpC

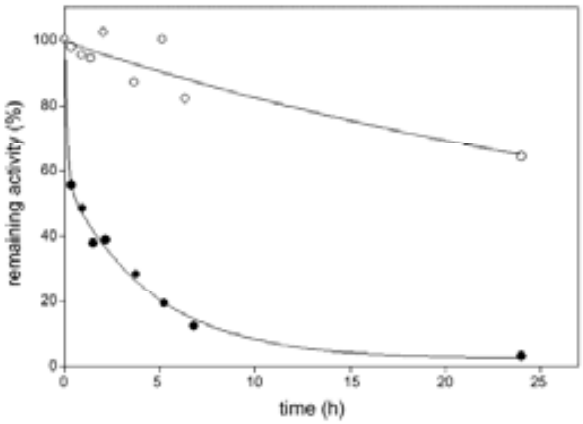

Fig. 2. Stability of β-galactosidase NF795gpC at 4 °C and pH 10.1 (●) or pH 9.5 (○).

was relatively stable during the whole process at pH 9.5, reaching an immobilization yield of 80%, and retention of 50% of the offered activity into the immobilized derivative.

3.3. Activation of engineered β-galactosidase

The enzyme derivatives prepared according to the above procedure were tested for their ability to give a signal proportional to their activation in presence of sera samples containing anti-HIV antibodies. The standard β-galactosidase activity assay using ONPG as substrate [15] was modified according to Section 2 indications. The activity of the immobilized derivative of *E. coli* β-galactosidase NF795gpC was measured in presence and absence of a model immune serum (60.0038 in Ref. [7]). Fig. 4 shows the response against the same serum concentration of both the immobilized preparation and the free enzyme. The activity measurements were performed by adding simultaneously the enzyme to the substrate and serum or buffer solutions. The maximum slope (proportional to β-galactosidase activity) was

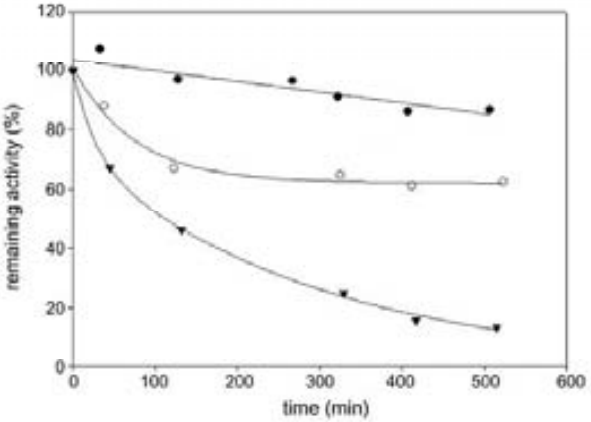

Fig. 3. Immobilization progress of β-galactosidase NF795gpC. (●) Activity evolution of the blank. (○) Suspension activity, accounting for the enzymatic activity in the supernatant plus the one immobilized onto agarose. (▼) Activity of the remaining protein in the supernatant.

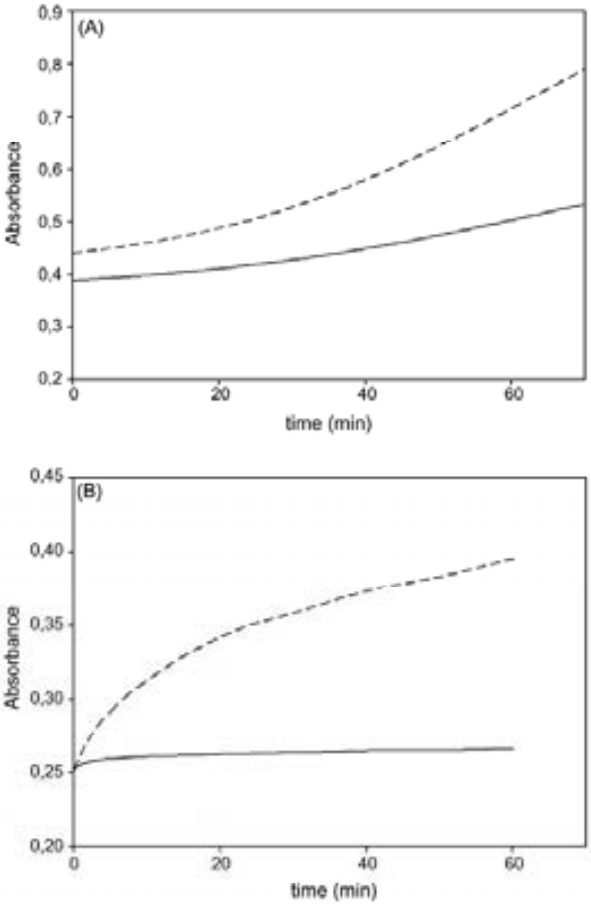

Fig. 4. Activity assay of the immobilized (A) and soluble engineered β-galactosidase (B) in presence (dashed line) and absence (continuous line) of a serum containing anti-HIV antibodies. 0.038 AU/mL was employed in all cases.

obtained as initial rate for the free enzyme (Fig. 4B), demonstrating that no previous incubation is required. As it can be seen in Fig. 4A, the reaction behavior was different for that of the immobilized enzyme. It could be due to a chemical modification of the enzyme leading to kinetic changes or to diffusional limitations affecting ONPG and antibodies mass transfer into the pores of the biocatalyst. Nevertheless, the difference in absorbance (or raised color) after 60 min was high enough to allow the discrimination between immune and non-immune sera.

The immobilized allosteric enzyme was shown to be stable after storage at 4 °C, maintaining 100% activity after 1 month. In order to check its applicability in field measurements, the immobilized allosteric enzyme stability at 37 °C was determined. As it can be seen (Fig. 5), the immobilized enzyme was more stable than the soluble one, maintaining around 80% of the initial activity after 5 h at 37 °C. The immobilized derivative was almost fully stable after 1 h incubation at this temperature. This time period is long enough to perform measurements at environment temperature without significant loss of activity (see Fig. 4A). This result is of especial significance for the development of a

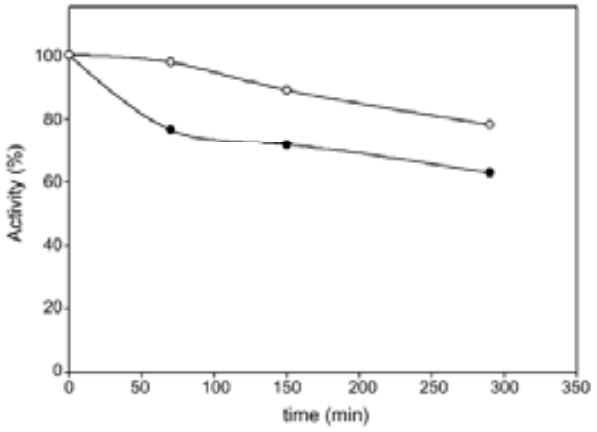

Fig. 5. Stability of engineered free (●) and immobilized (○) β-galactosidase at 37 °C.

biosensing methodology based on the response of immobilized allosteric enzymes.

The activation factor of the β-galactosidase sensors depends on the antibody concentration [8] which is expected to be different for each sample. A pool of HIV-infected patients' sera was employed in the proposed assay at different dilutions with the purpose of determining the titre conducting to a maximum response (saturation). The obtained data are presented in Fig. 6 as activation factor (100% in absence of serum) versus the serum/enzyme ratio (serum microliters per β-galactosidase activity unit). Although a higher activation degree can be achieved by using the free enzyme (Fig. 6), the immobilized preparation was able to reach values around 200% when volumes of serum above 300 000 μL serum/activity unit are employed. The sensitivity threshold of the immobilized enzyme is six times higher than the free enzyme one. Nevertheless, this sensing signal is good enough to allow a robust diagnosis of HIV-infection through the determination of antibodies in sera, with sensibility values comparable to that shown by standard immunoassays [7].

The general application of the proposed assay was tested using sera from different patients, the serum of a non-infected

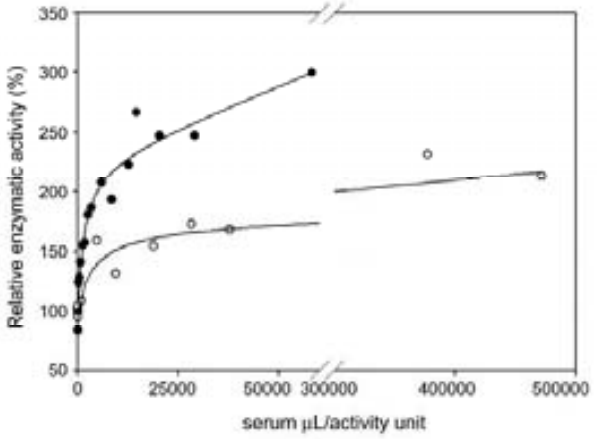

Fig. 6. Saturation curves using a pool of HIV infected patient sera. Soluble (●) and immobilized (○) engineered β-galactosidase.

Table 1
Activation values obtained with several HIV-infected patient sera. For comparison, data of standard HIV-1/2 test for each serum is included

Serum no.[a]	Activation factor (%)	HIV-1/2 test (AxSYM)[a,b]
60.0003	280.5	20.05
60.0006	183.3	19.98
60.0015	400	24.30
60.0022	280.5	41.11
60.0038	280.5	21.38
60.0043	220.6	24.48
60.0026	200.5	31.13
60.0011	198	25.79
60.0017	152.4	11.14
60.0030	218.6	24.53
60.23012	**108**	**0.30**

A control with a serum from a non-infected patient is remarked in bold.
[a] According to Ref. [7].
[b] AxSYM readings above 1 are considered positive, and readings below 1 are HIV-1/2 negative.

patient being included as a control. The results, presented in Table 1, confirm the activation of the immobilized enzyme in presence of the analytes, discriminating between infected and non-infected serum samples. For comparison, data concerning the sera titre measured by the commercial HIV-1/2 test have been reproduced from Ref. [7]. As can be seen, there is a qualitative agreement with the proposed immobilized enzyme method.

The potential of the proposed biosensing method allows envisaging the use of immobilized allosteric engineered *E. coli* β-galactosidase NF795gpC as the biological part of a future biosensor. The principle, illustrated in Fig. 7, consists of a suitable support containing the immobilized enzyme that has to be exposed to the problem serum and a control sample. After addition of the ONPG solution the target antibodies are to be detected by the color ratio between a positive sample and the control.

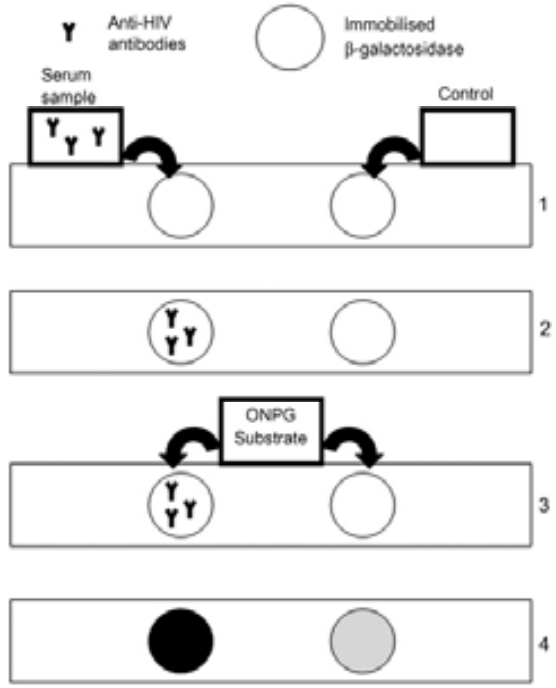

Fig. 7. Scheme of the operational principle for the proposed biosensing method.

This methodology is expected to be useful for field measurements to be managed by non-skilled personnel, for the qualitative determination of anti-HIV antibodies-containing samples.

The reported principle has to be developed in future research taken into account some other possible substrates or detection systems which could improve the final design of the biosensing device.

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References

[1] Thevenot DR, Thot K, Durst RA, Wilson GS. Electrochemical biosensors recommended definitions and classification. Biosens Bioelectron 2001;16:121–31.
[2] Ferraz RM, Vera A, Aris A, Villaverde A. Insertional protein engineering for analytical molecular sensing. Microb Cell Fact 2006;5:15.
[3] Villaverde A. Allosteric enzymes as biosensors for molecular diagnosis. FEBS Lett 2003;554:169–72.
[4] Benito A, Feliu JX, Villaverde A. Beta-galactosidase enzymatic activity as a molecular probe to detect specific antibodies. J Biol Chem 1996;271:21251–6.
[5] Ferrer-Miralles N, Feliu JX, Villaverde A. Molecular mechanisms for antibody-mediated modulation of peptide-displaying enzyme sensors. Biochem Biophys Res Commun 2000;275:360–4.
[6] Ferraz RM, Aris A, Martínez MA, Villaverde A. High-throughput, functional screening of the anti-HIV-1 humoral response by an enzymatic nanosensor. Mol Immunol 2006;43:2119–23.
[7] Ferrer-Miralles N, Feliu JX, Vandevuer S, Müller A, Cabrera-Crespo J, Ortmans I, et al. Engineering regulable *Escherichia coli* beta-galactosidases as biosensors for anti-HIV antibody detection in human sera. J Biol Chem 2001;276:40087–95.
[8] Ferraz RM, Aris A, Villaverde A. Profiling the allosteric response of an engineered beta-galactosidase to its effector, anti-HIV antibody. Biochem Biophys Res Commun 2004;314:854–60.
[9] Nuwayhid NF. Laboratory tests for detection of human immunodeficiency virus type 1 infection. Clin Diagn Lab Immunol 1995;2:637–45.
[10] Hashida S, Hashinaka K, Ishikawa S, Ishikawa E. More reliable diagnosis of infection with human immunodeficiency virus type 1 (HIV-1) by detection of antibody IgGs to pol and gag proteins of HIV-1 and p24 antigen of HIV-1 in urine, saliva, and/or serum with highly sensitive and specific enzyme

immunoassay (immune complex transfer enzyme immunoassay): a review. J Clin Lab Anal 1997;11:267–86.
[11] Gurtler L. Difficulties and strategies of HIV diagnosis. Lancet 1996;348:176–9.
[12] Tegbaru B, Messele T, Wolday D, Meles PH, Tesema D, Birhanu H, et al. Evaluation of rapid HIV test kits on whole blood and development of rapid testing algorithm for voluntary testing and counseling centers in Ethiopia. Ethiop Med 2004;42:267–76.
[13] Ferraz RM, Aris A, Villaverde A. Enhanced molecular recognition signal in allosteric biosensing by proper substrate selection. Biotechnol Bioeng 2006;94:193–9.
[14] Ullman A. One step purification of hybrid proteins which have beta-galactosidase activity. Gene 1984;29:27–31.
[15] Miller JH. Experiments in molecular genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1972.
[16] Guisán JM. Aldehyde–agarose gels as activated supports for immobilization–stabilization of enzymes. Enzyme Microb Technol 1988; 10:375–82.
[17] Álvaro G, Fernández-Lafuente R, Blanco RM, Guisán JM. Immobilization–stabilization of penicillin G acylase from *E. coli*. Appl Biochem Biotechnol 1990;26:181–95.
[18] Blanco RM, Guisán JM. Stabilization of enzymes by multipoint covalent attachment to agarose–aldehyde gels. Borohydride reduction of trypsin–agarose derivatives. Enzyme Microb Technol 1989;11:360–6.
[19] Guisán JM, Fernandez-Lafuente R, Rodriguez V, Bastida A, Blanco RM, Álvaro G. Enzyme stabilization by multipoint covalent attachment to activated pre-existing supports. In: Van den Tweel WJJ, Harder A, Buitelaar RM, editors. Stability and stabilization of enzymes, 47. Amsterdam: Elsevier Science Publ.; 1993. p. 55–62.

4. DISCUSSION

4.1. PROTEIN NF795GPC AS A MOLECULAR BIOSENSOR

4.1.1. SENSING OPTIMIZATION

In a previous study by Ferrer-Miralles et al (Ferrer-Miralles, Feliu et al., 2001), a series of hybrid recombinant β-galactosidase-based proteins was designed and synthesized. Different sized peptides from the protein gp41 of HIV, including the most important antigenic peptides (P1 and P2), were inserted in two different permissive sites of the protein. Most of the proteins with insertions in between amino acids 795 and 796 of β-galactosidase, called NF795gpA, NF795gpC and NF795gpD, presented higher enzymatic activity than those in between amino acids 278 and 279. Moreover, analysis of surface accessibility through the three-dimensional structure analysis of those proteins showed that NF795gpC presented the best surface accessibility of its inserted peptide P1, agreeing with the activation experiments on which this protein was the best responder to the presence of specific antibodies. This enzyme was selected for a large scale comparison of the modulation assay with a Food and Drug Administration-approved automated standard HIV detection method (AxSYM). There was a 94% of agreement between both tests; only two of the HIV-infected patients analyzed resulted as false negative and one negative serum gave a positive result (table 7). Interestingly, it presented the highest response of the negative set in the standard assay. After a deeper analysis of anti-HIV antibodies specificity from those false negative sera, the lack of specific anti-P1 antibodies and the presence of only antibodies specific to the epitope P2 were observed (Ferrer-Miralles, Feliu et al., 2001). These results confirm the potential of recombinant-epitope displaying β-galactosidases as molecular sensors for the detection of specific antibodies in human sera (Ferrer-Miralles, Feliu et al., 2001).

In spite of all these promising previous results, a low relation signal: background was obtained in these initial analyses. The maximum value of NF795gpC activation was close to 250%, a value too low to develop an optimal sensor device. This result was similar to the upper threshold observed previously to this type of biosensors (Ferrer-Miralles, Feliu et al., 2000), calculated with the correlation between the maximum activation factor of

different protein-only biosensors (β-galactosidase, β-lactamase and alkaline phosphatase) and the relative Kcat value (Ferrer-Miralles, Feliu et al., 2000). Moreover, proteins with lower Kcat value after protein engineering produced a higher activation factor.

TABLE 7. HIV1/2 AxSYM method compared with NF795gpC enzymatic modulation in a panel of 61 human serum samples. +++, 150 ≤ relative activity < 300; ++, 130 ≤ relative activity < 150; +, 110 ≤ relative activity < 130; -, relative activity < 110. ND, not done. AxSYM reading above 1 are considered positive, and readings below 1 are HIV1/2-negative. Adapted from Ferrer-Miralles et al(Ferrer-Miralles, Feliu et al., 2001).

Serum	HIV1/2 AxSYM	NF795gpC	Serum	HIV1/2 AxSYM	NF795gpC
60.0001	28.08	+	60.0002	0.41	-
60.0003	29.05	+++	60.0008	0.46	-
60.0004	32.90	+	60.0009	0.64	-
60.0005	26.20	+++	60.0020	0.74	-
60.0006	19.98	+++	60.0044	0.30	-
60.0007	24.85	++	161198 01	0.34	-
60.001	35.30	+++	161198 02	0.55	-
60.0011	27.59	+++	161198 03	0.52	-
60.0012	26.58	+++	161198 04	0.42	-
60.0013	21.47	+++	161198 05	0.51	-
60.0014	34.43	+++	161198 06	0.49	-
60.0015	24.30	+++	161198 07	0.50	-
60.0016	17.61	+	161198 08	0.54	-
60.0017	11.14	+	161198 10	0.53	-
60.0018	18.96	-	161198 11	0.60	-
60.0019	23.70	+++	161198 12	0.30	-
60.0021	30.92	+++	161198 13	0.57	-
60.0022	41.11	++	A8039 01	0.78	+
60.0023	24.78	+++	A8039 02	0.35	-
60.0024	24.87	+++	A8039 03	0.47	-
60.0025	35.60	+++	A8039 04	0.47	-
60.0026	31.13	+++	A8039 05	0.50	-
60.0027	14.81	+	A8039 06	0.42	-
60.0028	15.07	++	6022956	0.33	-
60.0029	14.60	-	6022982	0.29	-
60.003	24.53	+++	6023012	0.30	-
60.0038	21.38	+++	6117482	0.33	-
60.0039	21.91	+++	6117488	0.39	-
60.004	15.00	++	6210075	0.30	-
60.0041	31.90	+++			
60.0042	31.49	+			
60.0043	24.48	+++			
Anti-P1	ND	+++			

It has to be taken into account that a higher concentration of protein does not result in a higher response in presence of antibodies. In the first publication presented here, a deep study of the optimal concentrations of enzyme and substrate was done, revealing that the concentrations of both enzyme and substrate were critical for generating enhanced biosensing signals. Previously, a similar study was done by Cazorla et al but using proteins with one or more inserted fragments of FMDV (Cazorla, Feliu et al., 2002). In this case, a protein with only one copy of the epitope per monomer (called JX795A), as well as our case with the protein NF795gpC containing one copy per monomer but of a fragment of HIV, rendered a signal of 250%. However, when a protein with two copies of the insertion per monomer was used, an activation factor up to 500% was obtained (Cazorla, Feliu et al., 2002).

Topographical maps of the enzymatic response combining different concentrations of protein and substrate were constructed (Paper 1, Figure 3). In them, the obtained sensing signal was represented as different colours depending on the percentage of response. First, a comparison between topographical maps of NF795gpC protein and wild type β-galactosidase was done to evaluate the impact of the peptide accommodation on the enzyme performance (Paper 1, Figure 3E and 3F), revealing that the peptide insertion dramatically affected the activity of the enzyme. However, when a specific antibody was used in the assays, a clear increase of activity was observed (Paper 1, Figure 3D). So, the insertion would have instead enhanced the molecular flexibility required for signal transduction and also provided an allosteric-binding site for molecular sensing in a sensitive surface of the enzyme. Flexibility of allosteric biosensors was also measured when comparing the time-course of the reaction between the enzyme in absence of antibody, which after 10 minutes of reaction was no further processing substrate, and enzyme in presence of antibody, which arrived to a maximum of response only after 30 to 50 minutes (Paper 1, Figure 2). The flexibility of the enzyme would allow conformational modifications when the binding of the specific antibody occurs, permitting an improvement of the activation of the sensor.

In a step further, and to evaluate the possible effect of the low antibody concentration on the sensing assay, we compared activation factors obtained with a low and a high

concentration of antibody (Paper 1, Figure 3B and 3G). Topographical maps obtained in both cases showed a similar pattern, excluding the antibody concentration as a topographical definer and confirming enzyme and substrate concentrations as major controllers of the allosteric profile.

The amount of antibody bound to NF795gpC, measured through an indirect ELISA, and the resulting activation factor obtained by the enzymatic assay (Paper 1, Figure 1) were compared. It was observed that the amount of antibody giving the 50% of the maximal response in the enzymatic assay was higher than the antibody bound in the ELISA assay corresponding to the 50% of the total antibody bound. This fact indicated a possible enhancement of activity due to the simultaneous binding of effector antibodies to more than one monomer of the protein. Thus, a molar excess of the antibody might stimulate the allosteric signal. In fact, an enhanced activation when more than one effector molecule binds the tetrameric enzyme was previously observed (Alcalá, Ferrer-Miralles et al., 2002). However, the molar excess of antibody would also promote oligomeric complexes by cross-linking the enzyme. The possible effect of the formation of oligomeric complexes was studied using a secondary antibody (Paper 1, Figure 4). An enhancement of the activation factor by the formation of those complexes was observed. It probably occurred by the modulation of the adaptive binding to the receptor site and the consequent conformational signal.

Previous studies of interaction between protein and antibody suggested that some proteins need the bivalent capacity of the antibody to be activated whereas others can be activated only by the Fab fragment or monovalent antibodies (Feliu, Ferrer-Miralles et al., 2002). Here, the deep exploration of the biosensing mechanics suggests that multivalent antibody binding and the consequent enzyme network formation enhance allosteric activation (Paper 1, Figure 4, and Table 1).

Analysis of the crystal structure of β-galactosidase(Jacobson and Matthews, 1992) revealed that the 794-803 loop did not directly contribute to the shape of the active site but interacted with residues in this site (Ferrer-Miralles, Feliu et al., 2001). Furthermore, substitutions for Gly-794 affect the binding of substrates (Martinez-Bilbao and Huber,

1996). So, it is not unexpected that peptide insertions in this position affect kinetic constants. In fact, previously developed β-galactosidases (Ferrer-Miralles, Feliu et al., 2001) containing HIV-peptides had Kcat and Km values strongly affected by peptide insertion in an apparently opposite fashion, Km value increasing depending on the peptide size (Ferrer-Miralles, Feliu et al., 2001; Ferrer-Miralles, Feliu et al., 2000). The three-dimensional model of NF795gpC obtained by comparative modelling computational analysis (Ferrer-Miralles, Feliu et al., 2001) indicates that some portions of the insertion interact with the residues Asn-102 and Trp-999, important for substrate binding. Unsuccessful approaches avoid the crystallization of this protein as well as the parental β-galactosidase (Jacobson and Matthews, 1992).Moreover, the local secondary structure of the inserted peptide, which might induce a local rigidity affecting to the conformation of the protein in the vicinity of the active site, could have also an effect to the substrate binding.

Substrate binding can be influenced by several characteristics of the protein but it could be also influenced by the type of substrate. Since the modulation of substrate processing rate is the sensing signal itself, the specific substrate selected to determine the signal could be relevant to the final sensing data. So, a further analysis of the reactivation of the protein NF795gpC was done using different types of substrates, with the aim to improve the resulting activation values and to determine which are the best substrates to do it.

An analysis of six different β-galactosidase substrates was done in the second publication presented in the results. The objective was to define the main characteristics of every substrate regarding to sensitivity, activation range and sensor response. Since every substrate had a different molecular size and different properties it was not unexpected to find kinetic differences between them when the sensor was analyzed.

The first substrate tested was X-gal, being quickly discarded due to the instability of the protein in the buffer required for the X-gal dissolution. Lactose, the natural substrate of the protein, was tested since there are commercial amperometric (personal glucose blood tester) and colorimetric tests to determine the lactose hydrolysis product, glucose. First approaches with the amperometric system revealed slight problems

regarding to the sensing signal. Experiments with a monoclonal anti-P1 antibody produced 57.6 ± 13.3 mg/dL of glucose; however, the protein without antibody generated a value of glucose under the detection limits. Since human sera contain high amounts of glucose, different concentrations of both protein and substrate were tested similarly to the previous work with ONPG (Paper 1), trying to overcome sufficient basal levels of product to do the measures. However, only those HIV-immunoreactive sera gave a slight response. The lack of a detectable signal coming from the protein without antibodies did not allow obtaining a quantitative result. On the other hand, the glucose colorimetric test using the monoclonal anti-P1 antibody gave a maximal result close to 200%, comparable to those obtained in the initial studies with β-galactosidase and with ONPG (Benito, Feliu et al., 1996). Nevertheless, again higher concentration of glucose in sera masked the small amount of glucose produced by the protein. Hence, lactose was also discarded for further assays.

We comparatively analysed chromogenic (ONPG, CPRG), luminogenic (GalactonR) and fluorometric (FDG) substrates. Activation factor was first determined with different concentrations of specific monoclonal antibodies for each substrate (Paper 2, Figure 1) and the resulting plots were compared. Clearly, the activation profile was influenced by the substrate used and the equation constants on each case were also different (Paper 2, Table 1). The equation values were obtained as theoretical, constraining to 100% the parameter regarding to the minimal activation factor that could be obtained in absence of antibody, and real, where no constrain was applied. In spite of observing a different behaviour for each substrate, in all of them the activation of the sensor was linear at low antibody concentrations and it was modified at higher concentrations. The extent of linear range was determined, obtaining the widest response with the substrate CPRG. Moreover, among all substrates, ONPG was considered as the most sensitive because of its lower detection limit (Paper 2, Table 1).

Comparison of real and theoretical results (Paper 2, Table 1) showed big similarities for all the substrates excepting for CPRG, where the real basal activity (background) was of 61.26%. This value could represent an important variability in the sensing data for low analyte concentrations. Taking into account all obtained values for the substrates,

namely sensitivity, signal-background ratio, range of response and sensibility, chromogenic substrates were selected as the most promising for further experiments.

Then, several sera assays were done with CPRG and ONPG and again equation parameters were calculated (Paper 2, Figure 2 and Table 1). The activation profile was slightly different from that one obtained with monoclonal antibodies; however the relative properties of both substrates were maintained. CPRG was the substrate with a higher activation factor and the substrate with the widest response. Regarding the detection limits, in this case, CPRG detected the lower dilution of sera. Again, CPRG presented a poor adjustment of the basal value to the theoretical value of 100%, however it was better than the previously obtained with monoclonal antibodies. Both topographical maps of the sensing reaction with CPRG and ONPG were also done (as in paper 1), allowing the comparison of the optimal concentrations of protein and substrate giving the maximum response (Paper 2, Figure 3). Areas allowing amplified sensing signals were not coincident between both substrates, confirming different properties between them. Moreover, when those topographical maps were done with a pool of sera, these sensing areas were found more widely extended although with lower activation values. Results showed that peak activation values were higher when using CPRG than ONPG. Finally, to evaluate the reproducibility of these results, five different sera were tested for each substrate and again, higher activation values for CPRG were obtained (Paper 2, Figure 4). The fact that other authors have also used successfully CPRG to the detection of β-galactosidase activity but in blood samples (Pelisek J., Armeanu S et al., 2000), would represent an advantage if the biosensor has to be used in field measurements. The properties shown by CPRG are optimal for the development of this type of assay, especially because of the enhanced signal: background ratio.

4.1.2. ALLOSTERIC SENSING BY IMMOBILIZED NF795GPC

For many diagnostic applications in both animal an human health, it would be desirable the development of a solid phase biosensor version useful for field measurements where a large number of samples has to be analyzed and where sophisticated laboratory

resources are absent. The publication IV presented as a result in this thesis describes the immobilization of NF795gpC protein into a support to evaluate the allosteric sensing under these conditions.

Agarose was used as a support due to the previous experience of the group of Chemical Engineering of the Autonomous University of Barcelona, with who we collaborated in this study. The aim was to determine if the protein was reactivated when it was immobilized and if it was stable in this situation. Previous studies have been focused on the immobilization of non-allosteric β-galactosidases with biosensor purposes but in other supports such as gold layers (Ball, Puckett et al., 2003) or silicon surfaces (Betancor L, Luckarift HR et al., 2008).

4BCL agarose properties allowed the proper immobilization of the β-galactosidase due to its suitable porus size for the entrance of the protein and the convenient mass transfer during the reaction. First, a commercial β-galactosidase, 4BCL agarose (Paper 4, Figure 1) and 10BCL agarose were tested (not shown). Immobilization was based on a multicovalent attachment to the generated aldehyde residues on the support through the amine groups (Guisán, 1988; Alvaro, Fernandez-Lafuente et al., 1990). Due to the lower stability of the engineered enzyme NF795gpC under the alkaline conditions necessary for immobilization, kinetics of activity at different pHs were done (Paper 4, Figure 2). In spite of the optimal pH used in typical immobilization procedures (pH 10.1), a higher stability of the protein NF795gpC was observed at a pH 9.5, using this condition to develop the rest of assays (Paper 4, Figure 3).

Furthermore, stability of the immobilized enzyme was higher than that of the soluble version (Paper 4, Figure 5), allowing measurements at environment temperature without significant loss of activity. In general, this immobilization method allow the stabilization of proteins (Alvaro, Fernandez-Lafuente et al., 1990; Blanco and Guisan, 1989), as shown with penicillin G acylase (Alvaro, Fernandez-Lafuente et al., 1990) and proteases (Blanco, Bastida et al., 1991).

As previously described, the activation factor of the β-galactosidase sensors depends on the amount of antibody ((Benito, Feliu et al., 1996), so, different dilutions of a pool of

HIV-immunoreactive sera were used to determine the sera concentration giving a maximal response (Paper 4, Figure 6). Although a higher activation factor was achieved by the soluble protein, the immobilized preparation was able to reach activation values around 200%. In spite of the lower sensitivity obtained with the immobilized sensor than that obtained with the free one, the sensing signal was good enough to discriminate between infected and non-infected patients. Several HIV-1 immunoreactive sera were also tested with the immobilized version of the sensor to confirm the activation in presence of analyte, obtaining positive values between 150 and 400% (Paper 4, Table 1).

The biosensing principle described in this paper would allow comparing a serum sample with a control through a colorimetric assay in a support. The simple observation of a color change would give the result. Its simplicity would allow its use in regions where there is a lack of technologies and skilled personnel or as an initial test that could be done at home. Until now, no other allosteric enzyme has been immobilized to this aim and moreover, in a stable and functional way. In spite of the wide range of rapid HIV-tests that has been described recently (Greenwald, 2006) (see chapter 1.4.5); the simplicity of this one and the use of inexpensive materials would allow a better use of the social funds assigned to this issue.

4.1.3. CONSTRUCTION OF NEW ALLOSTERIC ENZYMES FOR ANTI-HIV ANTIBODY DETECTION

As it has been described above, protein NF795gpC exposes correctly the epitope P1 from gp41 of HIV-1, so only anti-P1 antibodies can be detected (Ferrer-Miralles, Feliu et al., 2001). In fact, the two false negative sera obtained when the comparison with the AxSYM system was done (Table 7, chapter 4.1.1), gave negative results to anti-P1 antibodies by ELISA test. However, the presence of anti-P2 antibodies was detected (Ferrer-Miralles, Feliu et al., 2001). Only a sensitivity of 94% was obtained previously with this protein (Ferrer-Miralles, Feliu et al., 2001). So, the development of additional allosteric proteins exposing other immunogenic epitopes of the virus, would improve the detection of several specific anti-HIV antibodies when used in combination (see

annex I). Using the new synthesized proteins together with the previously characterized NF795gpC, a more complete device could be developed. Hence, several immunogenic epitopes from HIV-1 were carefully selected (Annex I, Table 8), including fragments from the HIV proteins gp120, gp41, p24 and p17. Other groups have also used combination of different antigenic sites to the antibody detection and diagnosis of HIV infection, such as Hashida et al, who developed a highly sensitive and specific immune complex transfer enzyme immunoassay combining the detection of antibodies to RT (retro transcriptase), p17, p24 and p24 antigen(Hashida, Hashinaka et al., 1997; Hashida, Hashinaka et al., 1996).

The insertion of the new antigenic sites in permissive solvent exposed loops of β-galactosidase was done with the same cloning protocol as before (Ferrer-Miralles, Feliu et al., 2001), in order to compare the activation of these proteins and NF795gpC. Plasmid pJX795, a derivative from pJLA602 (Schauder, Blocker et al., 1987), was the vector used for the cloning processes, using its restriction site BamHI in between residues 795 and 796 to drive the epitope insertions. The new synthesized proteins were called JX795-MN, JX795-IIIB, JX795-B138, JX795-p24 and JX795-p17.

In a first approach, the best concentrations of both protein and substrate were selected to obtain the maximal signal: background ratio, similarly to the previous work with NF795gpC (Paper 1). However, CPRG was used as a substrate due to the higher activation values (Paper 2). Finally, after the analysis of the reaction kinetics of each protein, 30 minutes was selected as the optimal time at which the maximum response is reached. Once the conditions were established, sera from different patients were analyzed. A different activation factor depending on the protein was observed (Annex I, Figure 10). Three proteins containing fragments from gp41 and gp120 were clearly activated (NF795gpC, JX795-MN and JX795-IIIB), whereas those containing fragments from the capsid and matrix (p24 and p17) were less activated. As it has been described above, the HIV-1 envelope region (gp41/gp120) belongs to a region sensitive to the neutralization by antibodies, and this neutralization can be closely linked to the conformational changes induced by antibodies (de Rosny, Vassell et al., 2004). So, the observed activation in the proteins NF795gpC, JX795-MN and JX795-IIIB, could be due to

the effect of the antibodies on to the neutralizing region inserted in the proteins. Rosen et al studied the structure by nuclear magnetic resonance (NMR) of neutralizing peptides belonging to V3 loop of gp120 in strains IIIB and MN. Using two different specific antibodies and the peptides free in solution, changes in peptide conformation were observed, suggesting an induced fit produced by antibodies (Rosen, Chill et al., 2005).

Ferrer-Miralles et al analyzed the effect of the insertion of peptides with different sizes in the position 795-796 of the β-galactosidase (Ferrer-Miralles, Feliu et al., 2001). After analysis of kinetic constants and activities, an optimal length of 35 amino acids was found. Protein JX795-B138 is the only one containing a fragment lower than 35 amino acids (Annex I, Table 8), and in spite of being part of the gp120 protein (Morrow, Williams et al., 1992) and having a neutralizing epitope (de Rosny, Vassell et al., 2004), there was no activation of the enzyme (Annex I, Figure 10).

A low activation factor was obtained with sera in the enzymes containing fragments of the *gag*-derived HIV-proteins p24 and p17 (JX795-p24 and JX795-p17) (Annex I, Figure 10). Humoral immune response against p24 and p17 has been described to appear in early stages of HIV infection, moreover, its affinity to the epitope correlates with disease progression (Chargelegue, Stanley et al., 1995; Kozinetz, Matusa et al., 2005) making them suitable as prognosis markers (Janvier, Baillou et al., 1991). High levels of p17 antibodies correlate with slower progression to AIDS (Fiorentini, Marini et al., 2004). In fact, p17 and p24 have been used in HIV tests for an early detection of the infection in the *window* period. In this period anti-HIV antibodies production is just starting and normally they are under the detection limits of HIV-antibody tests (Hashida, Hashinaka et al., 1996). It has to be taken into account that as the disease progresses to AIDS, the level of antibodies to the HIV-1 gag proteins falls progressively (Allain, Laurian et al., 1991). So, the low activation of both proteins (JX795-p24 and JX795-p17) could be maybe due to the analysis of HIV-1 sera samples from patients in advanced stages of disease, where the level of anti-*gag* antibodies is low. However, the lack of information about the stage of disease on which are those analyzed patients, avoid the confirmation

of this hypothesis. Hence, further analysis of activation of these proteins would be of interest to know if there is recognition of early infection antibodies.

The sensor activation capacity of antibodies present in five different sera samples was analyzed using the newly synthesized proteins (Annex I, Figure 11). Clear differences of activation levels were observed between proteins, demonstrating that different patients had different specific antibody composition in serum.

Finally, linear regression analysis between activation values and $CD8^+$ (CD8), $CD4^+$ (CD4) T cell count and viral load (VL) were obtained (Annex I, Table 9 and 10). Good regression parameters (p and r) were obtained when JX795-B138 protein activation was compared with the level of $CD4^+$ T cells in sera ($p = 0.029$). This correlation was also maintained when data was divided into two groups depending on whether the patients followed or not antiretroviral therapy (Annex I, Table 10).

As it is described in Table 8 (Annex I), protein JX795-B138 contains a fragment from the CD4 binding site in gp120 (Morrow, Williams et al., 1992). To study the possible activation of JX795-B138 by $CD4^+$ T cells, a commercial soluble glycoprotein CD4 was used. Higher concentrations of the glycoprotein CD4 resulted in higher activation factors of the JX795-B138, although the activation values arrived only to 120%. However, no activation of the protein NF795gpC in the same conditions was observed (Annex I, Figure 12). So, it seems that the soluble CD4 glycoprotein is able to activate the protein JX795-B138.

Finally, those sera classified as false negative by NF795gpC protein (60.0018 and 60.0029 from Table 7) (Ferrer-Miralles, Feliu et al., 2001) were analyzed using the new enzymes. Protein JX795-MN, containing a neutralizing fragment from gp120 HIV-1-protein, responded positively to those sera generating sensing values of 262.8% and 151.4% respectively (not shown). So, the use of other immunogenic epitopes to the development of new allosteric HIV-1 sensors, allow the detection of other specific anti-HIV-1 antibodies and improve the previous sensitivity obtained with the protein NF795gpC.

4.2. PROTEIN NF795GPC AS A TOOL FOR THE SCREENING OF SPECIFIC ANTIBODIES

As explained in the introduction, HIV has several mechanisms to escape from the immune system, such as changes in the glycosilation pattern or amino acid substitution producing conformational changes in specific epitopes (Wei, Decker et al., 2003; Park, Gorny et al., 2000; Zhang, Bouma et al., 2002). This conformational camouflage indicates a high flexibility of the region gp120-gp41 (Myszka, Sweet et al., 2000). In the context of this plasticity, neutralization of HIV-1 might be closely linked to the antibody-promoted conformational perturbation of the epitope itself and surrounding areas of influence (de Rosny, Vassell et al., 2004). Moreover, the responsible for structural modifications is the antibody and not specific features of particular gp120 epitopes (Kwong, Doyle et al., 2002). Protein NF795gpC contains the peptide P1 from the env-derived protein gp41, which is one of the most neutralizing epitopes of the virus (Broliden, von Gegerfelt et al., 1992), in its native helical conformation (Ferrer-Miralles, Feliu et al., 2001). One copy of this peptide is exposed in every subunit of the protein, close to the active site of the enzyme. Activity of the engineered β-galactosidase NF795gpC is affected by the insertion, however, adaptive interaction with specific antibodies induces conformational changes leading to an activation dependent on the antibody concentration (Benito, Feliu et al., 1996). Here, the sensor is proposed to be useful in the evaluation of the structural impact of HIV-1 immune sera binding on the antigenic segment of gp41 and as a tool to study the properties of the HIV-neutralizing antibodies.

First, the correlation between the activation factor and antibody binding (measured by ELISA and AxSYM) (Paper 3, Table 1) was analyzed. The comparison of the activation factor induced by immune and non-immune sera of patients as a whole and the antibody titer gave a good correlation. Also, activation of NF795gpC was linearly dependent on the effector antibody concentration, as it was previously described (Benito, Feliu et al., 1996). However, when the analysis was done taking into account only the immune sera, no correlation was observed. This indicated that the mere interaction between the protein and the antibody is not enough to generate the sensing signal. Maybe different types of antibodies have different abilities in changing epitope

conformation. In fact, in studies by BIAcore technology with two monoclonal anti-gp41 antibodies with indistinguishable affinity constants, different level of sensor activation was observed (Paper 3, Table 2).

An analysis of the anti-gp41 specific antibodies subpopulations from sera of HIV-infected patients was done to identify which functional antibodies were promoting the activation of the sensor. The use of the protein NF795gpC as the antigen to develop several indirect ELISAs with secondary antibodies recognizing IgA, IgE, IgM, IgG1, IgG2, IgG3 and IgG4 antibodies, allowed the determination of the amount of specific antibodies of each isotype in sera. This data was compared with the sensor activation. The results showed a good correlation with the levels of IgG4 subpopulation ($p = 0.023$, $n = 15$) (Paper 3, Table 3). Moreover, the increase in the number of sera samples resulted in a better significance of the correlation ($p = 0.001$, $n = 55$). This indicated that among all the types of antibodies, IgG4 were the most involved in sensor activation, suggesting an especial ability in promoting conformational perturbations in the target antigenic site. No correlation of the activation factor with clinical parameters (viral load, CD4+ and CD8+ T cell count) was found (Paper 3, Table 3).

IgG4 isotype has an especial structure. Briefly, it is composed by a short bridge and it does not have covalent interaction between heavy chains. The main function is the interference with immune inflammation induced by complement-fixing antibodies or by IgE antibodies (normally in allergies and helminth infection) (Aalberse and Schuurman, 2002). IgG4 acts as a blocking antibody in allergic diseases and its titer increases during infection by viruses such as measles (Isa, Martinez et al., 2006; Isa, Martinez et al., 2002). And it is also important in pancreatitis, being used as a clinical marker(Hamano, Kawa et al., 2001). Regarding to HIV, it has been described that this type of antibodies in slow progressors of AIDS is correlated with the level of CD8+ T cells, while in rapid progressors, total IgG4 correlates with the level of CD4+ T cells (Abbas, Vasilescu et al., 2005). IgG4 against gag proteins (Klasse, 1996), but also against env proteins has been detected. In fact, most of the few IgG2-IgG4 reactions occur with the peptide HIV env 604-625 (Klasse, Blomberg et al., 1990), the same as it is inserted in the protein NF795gpC (Ferrer-Miralles, Feliu et al., 2001). Moreover, IgG4 response tends to appear

after prolonged or iterated exposure to an immunogen, being more common in HIV infected sera from patients in late clinical stages of HIV infection (Ljunggren, Broliden et al., 1988).

It is widely known that HIV generates a major neutralizing response of specific IgG1 antibodies (Klasse, Blomberg et al., 1990). The structure of IgG1 and IgG4 isotype was compared (Aalberse and Schuurman, 2002) observing clear differences between them, but a similar antigen cross-linking capacity when using mouse-human monoclonal chimeric IgG1 and IgG4 antibodies. However, polyclonal IgG4 did not cross-link two antigens (Aalberse and Schuurman, 2002). IgG4 seemed to be less neutralizing than IgG1 and IgG3 subtypes (Miranda, Duval et al., 2007).

In fact, IgG4 antibodies have been already used commercially as antiretroviral agents, for example, in the patent WO/2005/063282, IgG4-gp41 mimetibodies were synthesized to function as antagonists of gp41 (2005c). IgG4 have been also used as a method to reduce the viral load in HIV (patent 20070026441). TNX-355, a humanized IgG4 version of the anti-CD4 monoclonal antibody (mAb) 5A8, blocks fusion events that occur post-attachment of gp120 to CD4 (Burkly, Olson et al., 1992).

In a step further, the impact of antiretroviral treatment on the ability of sera to activate NF795gpC was analyzed (see annex II). It is known that HIV replication is required for an efficient production and maintenance of significant titers of anti-HIV IgG antibodies in sera (id-Peralta, Grangeot-Keros et al., 2006). As a consequence, the administration of antiretroviral drugs has a dramatic, negative impact of the immunoglobulin composition, titer and avidity in target subjects (id-Peralta, Grangeot-Keros et al., 2006; Voltersvik, Albrektsen et al., 2003).

As in paper 3, the activation factor of NF795gpC was determined with a series of sera (Annex II, Table 1) and antibody composition (IgA, IgE, IgM, IgG1, IgG2, IgG3 and IgG4) was studied through indirect ELISA procedures using NF795gpC as antigen (Paper 3). So, a parallel study of both binding and epitope conformation modification ability of antibodies was done. Now, the presence of an antiretroviral treatment was taken into account, determining possible correlations between activation factor of NF795gpC and

levels of each subpopulation of antibodies in both treated and untreated patients (Annex II, Table 2). Again, when sera from HIV-infected patients were analyzed, immunoglobulin IgG4 was the responsible for sensor activation. However, when sera from non-treated infected patients were analyzed, IgM became the main contributor of sensor activation ($p = 0.005$; Annex II, Figure 2A). As there were no significant differences between the titer of IgG4 and IgM ($p = 0.96$ and $p = 0.48$ respectively) on both treated and untreated groups of sera, we concluded that antiretroviral therapy (ART) produced an impact into the functionality of these antibodies. The induced fit promoted by IgM was lost during drug treatment at expenses of the conformational modification promoted by IgG4. Moreover, since the composition of antiretroviral drugs was variable (Annex II, Table 1) and because of the drop of antigenemia generated by the ART (Binley, Trkola et al., 2000), the change of contribution of specific types of antibodies could not be attributed to any specific drug and probably there is a functional adaptation of the humoral response. No correlation was observed between other clinical parameters (viral load, CD4 and CD8+ T cell count) and sensor signal.

Only some authors have studied the occurrence of specific IgM during HIV infection. There are specific IgM antibodies against gp41 HIV protein, although they seemed to disappear early after infection (Muller and Muller, 1988). An IgM immune response after injection of the vaccine NYVAC (gp120 fragment) was found, suggesting that IgM may have antiviral activity (Sheppard, Bates et al., 2007). So, the meaning of the observed correlation still needs further investigations.

A correlation near significance ($p = 0.09$; Annex II, Table 2) was also observed between IgE levels and activation factor. Despite not being important regarding statistical analysis, IgE subpopulation is related to HIV infection (see humoral response chapter). Elevated level of IgE in sera of HIV-infected individuals is a characteristic of HIV infection, and can also be used as a diagnostic tool (Fletcher, Miguez-Burbano et al., 2000; Zar, Latief et al., 2002). Anti-HIV-1 IgE of infected children, strongly inhibited HIV multiplication in culture, although the involvement of direct viral neutralization is not clear (Pellegrino, Bluth et al., 2002). HIV infection produce the induction of Th2 cells and synthesis of IgE (Becker, 2004). In allergic diseases, IgG4 antibody responses are often

associated with IgE antibody responses because both require the stimulation of Th2-type T-helper cells (Aalberse, Van Milligen et al., 1993). Despite these facts, further investigations are required to describe the exact role of IgE on biosensor operation.

All these results have described the functional changes of HIV-specific antibodies induced by the antiretroviral treatment. Hence, the use of enzymatic sensors to the detection of anti-HIV antibodies has been extended to the high-throughput analysis of antibodies present in sera samples.

Finally, further incorporation of the newly synthesized proteins (Annex I, Table 1) to complex sensing systems could also help to the screening of the antibodies relevant to the control of HIV infection.

5. CONCLUSIONS

1. Modulation of both enzyme and substrate concentrations can improve the sensing signal of NF795gpC protein reaching activation factors up to 1200% with sera of infected patients. Moreover, allosteric activation can be also enhanced by the multivalent antibody binding and the consequent enzyme network formation.

2. The use of different substrates implies a different sensing signal value, being influenced the sensitivity and the maximum activation values. In general, chromogenic substrates are the best substrates to develop enzyme biosensors. ONPG was the chromogenic substrate showing the lowest detection limit and CPRG generated the highest sensing signals.

3. Sensor activation by human, HIV immunoreactive sera is clearly dependent on IgG4 antibody subpopulation. However, analyses of patients that have not followed an antiretroviral drug treatment reveal an important activation effect of IgM antibodies. In presence of antiretroviral treatment, there is then a change in the functionality of the antiviral response, maybe inducing a functional dominance of IgG4 subpopulation.

4. Activation mediated by antibodies is conserved when the NF795gpC protein is immobilized on solid or semisolid supports. Furthermore, it is stable enough to perform measurements at environment temperature without significant loss of activity.

5. The simplicity and fastness of β-galactosidase sensing assay using chromogenic substrates can make the sensing assay useful for a high-throughput screening of a large number of samples. The fact that it can be immobilized maintaining the sensor capacity and improving its stability, could be useful to the development of solid-phase sensors for *in situ* measurements in places where there is a lack of skilled personnel and resources.

6. A wider range of antigenic sites inserted in the new biosensors allowed the detection of different types of HIV-specific antibodies.

7. Glycoprotein CD4 is able to modify conformationally the active site of the sensor JX795-B138.

6. ANNEXES

6.1. **ANNEX I**

TABLE 8. Sequences of the inserted peptides, peptide size and region of HIV which is exposed.

BIOSENSOR	SEQUENCE	PEPTIDE SIZE	REGION	HIV-1 PROTEIN	REFERENCE
NF795gpC	^{579}GIKQLQARILAVERYLKDQQLLGIWGCSGKLICTT^{613}	35	P1/P2	gp41	*(Ferrer-Miralles, Feliu et al., 2001)*
JX795-B138	^{421}KQFINMWQEVGKAMYAPP^{438}	18	CD4BS	gp120	*(Morrow, Williams et al., 1992)*
JX795-p24	^{196}AAMQMLKETINEEAAEWDRVHPVHAGPIAPGQMRE^{230}	35		p24	*(Janvier, Baillou et al., 1991)*
JX795-IIIB	^{271}INCTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQ^{305}	35	V3 (IIIB strain)	gp120	*(Broliden, von Gegerfelt et al., 1992)*
JX795-MN	^{299}INCTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQA^{333}	35	V3 (MN strain)	gp120	(Broliden, von Gegerfelt et al., 1992)
JX795-p17	^{2}GARASVLSGGELDRWEKIRLRPGGKKKYKLKHIVW^{36}	35		p17	*(Fiorentini, Marini et al., 2004)*

TABLE 9. Correlation parameters between activation factor of each protein and viral load (VL), CD4+ T cell (CD4) and CD8+ T cell (CD8) count regarding to the total of samples. Significant results are indicated in bold.

	VL		CD4		CD8	
	p	r	p	r	p	r
NF795gpC	0,1936	0.1747	0,3051	0.1382	0,5800	0.0755
JX795-MN	0,8996	0.0194	0,5139	0,0999	0,7165	0.0563
JX795-IIIB	0,2305	0.1824	0,5212	0.0982	0,1451	0.2233
JX795-P17	0,6144	0.0754	0,1209	0.2294	0,2155	0.1862
JX795-P24	0,1543	0.2184	0,8374	0.0318	0,7632	0.043
JX795-B138	0,4772	0.1100	**0,0291**	0.3293	0,5333	0.0977

TABLE 10. Correlation parameters between activation factor of each protein and viral load (VL), CD4+ T cell (CD4) and CD8+ T cell (CD8) count regarding to the treated and non-treated group of samples. Significant results are indicated in bold.

	VL		CD4		CD8	
Non-treated	p	r	p	r	p	r
NF795gpC	0,6420	0.1177	0,5058	0.1677	0,0574	0.4693
JX795-MN	0,4393	0.2468	0,6032	0.1673	0,8633	0.0590
JX795-IIIB	0,2859	0.3358	0,5366	0.1983	0,2090	0.4111
JX795-P17	0,9957	0.0017	0,9288	0.0290	0,7584	0.1051
JX795-P24	0,8284	0.0742	0,4739	0.2417	0,3970	0.3016
JX795-B138	0,9263	0.0317	**0,0461**	0.6105	0,1614	0.4789

	VL		CD4		CD8	
Treated	p	r	p	r	p	r
NF795gpC	0,4008	0.1988	0,3821	0.2066	0,4764	0.1690
JX795-MN	0,1307	0.3944	0,4869	0.1875	0,2511	0.3047
JX795-IIIB	0,0878	0.4403	0,4000	0.2260	0,7851	0.0741
JX795-P17	0,3799	0.2202	0,4434	0.1928	0,2478	0.2873
JX795-P24	0,2307	0.3176	0,9557	0.0151	0,9877	0.0042
JX795-B138	0,2001	0.3382	**0,0162**	0.5899	0,9686	0.0107

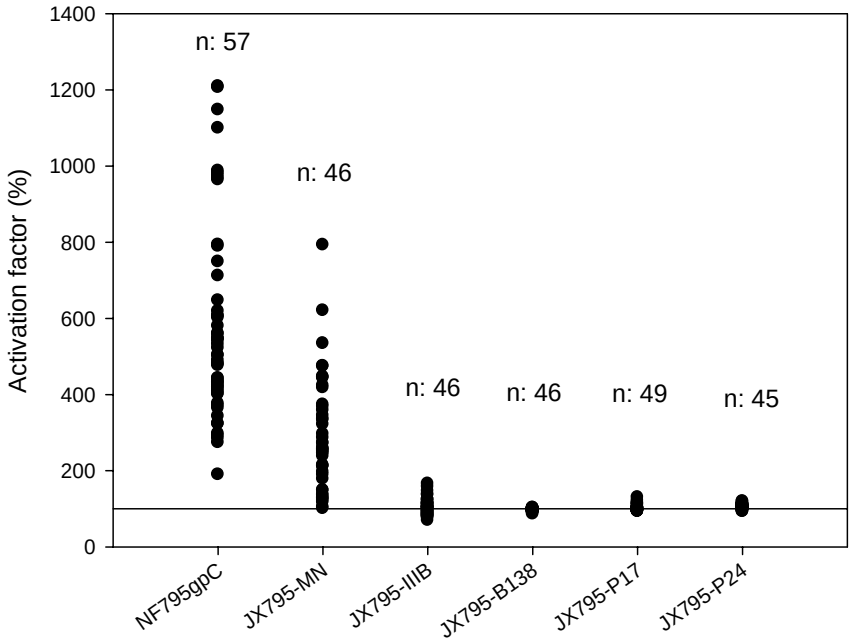

FIGURE 10. Raw activation factor values for each protein. A reference line gives the basal activation factor (100%). N: number of tested samples.

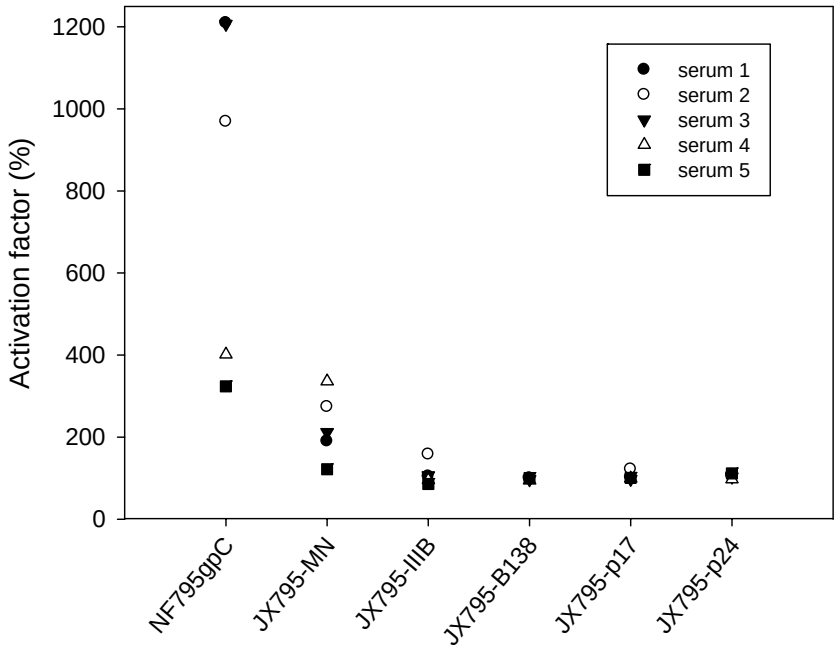

FIGURE 11. Activation of the proteins with 5 different HIV-1 infected sera.

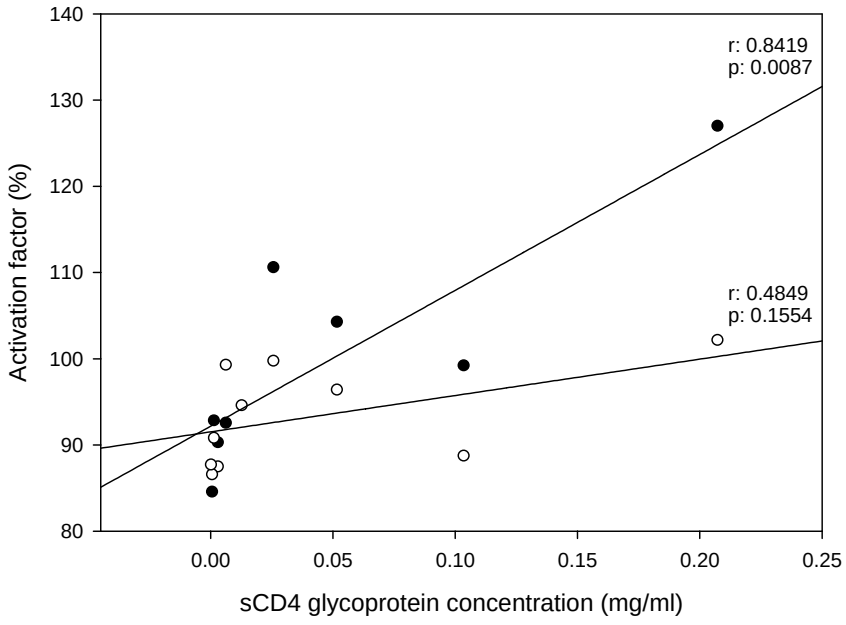

FIGURE 12. Activation of the sensor of the protein JX795-B138 (•) and the protein NF795gpC (º) in presence of a commercial soluble CD4 glycoprotein. This work was done in the Institute of Virology of the University of Saarland, under the supervision of Professor A. Meyerhans.

6.2. **ANNEX II**

ANTIRETROVIRAL THERAPY-INDUCED FUNCTIONAL MODIFICATION OF IGG4 AND IGM RESPONSES IN HIV-1-INFECTED INDIVIDUALS SCREENED BY AN ALLOSTERIC BIOSENSOR

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Antiretroviral Therapy-Induced Functional Modification Of IgG4 And IgM Responses In HIV-1-Infected Individuals Screened By An Allosteric Biosensor

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ABSTRACT

By using an enzymatic immunosensor that detects epitope-modifying anti-gp41 antibodies, we have identified an irreversible impact of antiretroviral treatment (ART) on the functionality of IgG4 and IgM specific antibodies in HIV-1-infected individuals. During ART, the prevalent induced fit promoted by IgM was lost at expenses of that promoted by IgG4, suggesting alternativeness in the neutralization potency of these antibody subpopulations. Since the particular ART used was irrelevant, the obtained data indicate that the drop in the viral load and the consequent lost of antigenemia are responsible for the observed functional adaptation of the humoral response.

Key words: antiviral drug; immunosensor; HIV-1; humoral response; gp41

INTRODUCTION

The humoral response against the human immunodeficiency virus-1 (HIV-1) infection has been long time considered of poor relevance regarding the progression of the infection. However, antibody-mediated viral neutralization and the consequent rising of evasive mutants have been proven as important contributors of HIV-1 dynamics in infected individuals[1,2]. In the case of HIV-1, the neutralization potency of antiviral antibodies directed to env products (gp41 and gp120) depends on the extent of structural perturbations caused by the antibody binding on the target epitope and surrounding areas[3,4]. This fact might be related to the high conformational flexibility of gp41-gp120 complexes at the virion surface that is required for the structural adaptation of both proteins to the CD4 receptor during cell attachment[5]. The neutralization potency of selected monoclonal antibodies can be predicted through monitoring the enthalpic and entropic changes in the ligand upon antibody binding as a reflect of the extent of induced fit[3]. However, such parameters cannot be obtained from whole sera and the spectrum of available neutralization assays remain as the only suitable methods for evaluation of neutralization capability[6].

We previously developed an engineered version of the *E. coli* β-galactosidase that displays the main immunodominant epitope from HIV-1 gp41, in a solvent-exposed area of the protein[7]. The modified enzyme (NF795gpC) responds allosterically to anti-gp41 monoclonal and polyclonal antibodies through conformational perturbations induced by the antibody on the enzyme's active site. The fact that specific antibodies generate an increase of the specific activity of the enzyme makes this enzyme a very efficient and sensitive 'protein-only' biosensor[8] for the screening of humoral anti-HIV-1 responses and consequently the detection of HIV-1 infection[7]. Recently[9], we have shown that different immunoglobulins in the sera on infected individuals do not act equally as effectors for the allosteric sensing, being the anti-HIV-1 IgG4 subpopulation the most efficient in the generation of the allosteric signal. This indicates that the 'induced fit' abilities of IgG4 are distinguishable from the rest of gp41 specific antibodies.

On the other hand, it is known that HIV-1 replication is required for an efficient production and maintenance of significant titres of anti-HIV-1 IgG antibodies in serum[10]. Consequently, the administration of ART has a dramatic, negative impact on the immunoglobulin composition, titre and avidity in treated subjects[10,11]. However, data about the molecular quality of anti-viral antibodies before and after treatment, and how the inhibition of viral replication might affect the neutralization potential of antibody subpopulations are not available. In this context, we have examined here the capability of different immunoglobulin classes to activate the allosteric sensor NF795gpC in sera from both untreated and treated patients, as well as the influence of ART on the functional quality of the humoral anti-HIV-1 response.

MATERIALS AND METHODS

Biosensor description and production

The antibody-sensing β-galactosidase enzyme NF795gpC displays the P1 immunodominant B-cell epitope of HIV-1 gp41 on a solvent-exposed position[7]. In this location, the accommodated viral peptide is accessible and fully immunoreactive. The binding of specific anti-P1 antibodies produces structural changes that result in detectable variations in the enzyme specific activity, easily detectable in a homogeneous assay. The hybrid enzyme NF795gpC becomes then an allosteric nanosensor[8] in which the P1 peptide acts as the specific receptor and antibodies as allosteric effectors. NF795gpC was produced in *Escherichia coli* BL26 strain through the thermosensitive expression vector pNF795gpC. After 4 hours of induction at 42ºC, protein was centrifuged and purified by conventional affinity chromatography[9]. The harvested protein was then dialyzed against Z buffer[12] and it was quantified by Bradford analysis[13].

Sera samples

A total of 57 HIV-1 infected human sera were used in this study (Table 1). Eighteen were from healthy individuals, 20 from HIV-1-infected patients who received ART without interruption for 55 weeks (mean; range 16-120), and 19 from HIV-1-infected patients who undergone structured treatment interruption for 30 weeks (mean; range 2-112 weeks). All the sera were tested for NF795gpC allosteric activation. Sera from 6 non-infected individuals were included in the analysis as negative controls.

Biosensing analysis

The enzymatic sensing assay was performed in 96-well microtiter plates by incubating 20 μl of 1:4 dilution of each serum in Z buffer (pH 7.0; 0.06 M Na_2HPO_4, 0.04 M $NaH_2PO_4H_2O$, 0.01 M KCl, 1 mM MgSO4 $7H_2O$) with 10 μl of 4.16 nM NF795gpC per well. After incubation for 1h at 25ºC, 40 μl of 1.66 mM chlorophenol red β-D-

galactopyranoside (CPRG, a lactose analogue rendering red hydrolysis products) in Z buffer were added. After 30 minutes of reaction, the absorbance was determined at 540 nm in a Labsystems IEMS plate reader. Percentages of enzyme activation were determined by referring to values obtained in absence of sera and used as a convenient numeric biosensing signal. All the experiments were performed in triplicate.

ELISA

For antibody class and subclass analyses, high binding Costar ELISA plates were coated with saturating concentrations of NF795gpC (7 pmol per well) and 100 µl of sera previously diluted in PBS at 1/500 were added to each well. Different types of anti-human secondary antibodies were used at optimal dilutions (anti-IgG, 1/2000 from Pierce; anti-IgG1, 1/500; anti-IgG2, 1/2000; anti-IgG3, 1/1000; anti-IgG4, 1/2000; anti-IgM, 1/2000; anti-IgA, 1/1000 and anti-IgE ,1/500 from Southern Biotechnology Associates, Inc.). The ELISA reaction was developed with 2, 2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) from Sigma and antibody binding measured at 405 nm. The determinations were performed in a minimum of 14 sera samples from all the cohorts. When the numerical study required more data for significance analysis, the determination of antibody composition was extended to a bigger number of samples. All the experiments were performed in triplicate.

Statistical analysis

Correlation between the activation levels of NF795gpC promoted by each sera sample and titres of the different types of immunoglobulins found in the same sera were done using the linear regression analysis tool from Sigma Plot v.10.0. The comparisons between activation levels in sera sets according to immunoglobulin titre and group of treatment were performed by the Kruskall-Wallis non-parametric statistical test. P values under 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

To better understand the anti-viral immune response in HIV-1-infected individuals and the role of ART in the humoral response, we determined which specific antibody populations are responsible for the sera-mediated NF795gpC sensor activation. For that, we explored, in the selected sera samples (Table 1), the correlation between the sera-induced increase of NF795gpC β-galactosidase activity[7] and the prevalence of different classes of antibodies reactive with the target B-cell epitope acting as sensor receptor, determined by indirect ELISA using NF795gpC itself as immobilized antigen[9]. Both analysis, then, detected specific antibodies directed against the gp41-P1 immunodominant epitope present in NF795gpC, regarding either epitope conformational perturbation or mere immunoreactivity respectively.

The profiles of biosensor signals promoted by the tested sera samples (Figure 1) were indistinguishable when comparing sera from ART patients and sera from non treated patients, as confirmed by a Kruskal-Wallis test (H=3.37, p= 0.06). Negative controls, namely sera from non infected individuals, showed activation factors not higher than 100% (not shown). When investigating possible linear correlations between activation factors and the amount of specific antibody classes, we found that, as described previously, the levels of allosteric activation of NF795gpC correlated significantly (*p*= 0.025) with the prevalence of gp41-binding IgG4 in sera (Table 2 and Figure 2B). This result indicates a main contribution of such IgG subclass in the modification of the gp41 B-cell epitope used as sensor receptor. However, when analyzing separately the sera pool from non treated individuals, the linear dependence observed previously between the sensor activation signals and IgG4 titres was completely lost. Instead, in absence of antiviral treatment, the occurrence of specific anti-gp41 IgM antibodies clearly determined the extent of NF795gpC activation (p=0.005; Figure 2 A). Since the composition of the ART was non homogeneous (Table 1), the shift in the contribution of specific immunoglobulin populations to the sensor activation could not be attributed to any specific drug. In addition, ART intervals were also diverse in the set of analyzed individuals. In those in which the treatment had been interrupted, viral load values were notably high, indicating the recovery of viral replication after interruption (Table 1).

Since both populations of sera (from patients receiving interrupted ART and continuous treatment) were statistically indistinguishable regarding sensor activation (p=0.48; not shown), the changes in the functionality of the antiviral response were therefore irreversible and did not depend on the actual rate of virus multiplication.

In summary, in naive HIV-1-infected individuals, antiviral IgM is the antibody class most efficient in the conformational modification of a model immunodominant B-cell epitope, the P1 segment of gp41. However, in individuals receiving ART, the induced fit promoted by IgM (if existing), is eclipsed by a functional dominance of IgG4. This dominance was evident when untreated and treated individuals were analyzed together (Table 2). Since the titres of specific IgG4 and IgM were not distinguishable when both groups were compared (p=0.96 and p= 0.48 respectively), the results shown in Table 2 indicate that ART produces a profound impact on the functionality of the humoral antiviral response, probably by either reducing the induced fit impact of IgM (and presumably, their neutralizing ability) or increasing that of IgG4 or both. Such effect is irrespective of both the specific drugs used in ART and the continuity of the treatment, and therefore, it does not depend on low viral replication or load. Therefore, the drop of antigenemia associated to ART, that specially affects envelope proteins irrespectively of the ART[14], is probably causing a functional adaptation of the humoral response in which IgG4 gains neutralization competence at expenses of IgM. No correlation was observed, as expected, with the rest of clinical parameters, namely VL, CD4+ and CD8+ T cell titres, which do not participate in the sensor activation (Table 2).

Finally, although being not significant, the correlation between IgE levels and activation factor in non-treated patients rendered a p value close to the significance limit (p= 0.099). Previous studies have observed a clear inhibition of HIV-1 multiplication by specific IgE under different conditions[15] and elevated levels of specific IgE are characteristics of the HIV-1 infection[16]. These observations strongly suggest an important role of this immunoglobulin in controlling the HIV infection and therefore, a possible role of this type of IgE in sensor activation might be clinically relevant but it would deserve further investigation.

Importantly, the results showed here stress the value of allosteric nanosensors as powerful tools for functional and dynamic screening of individual anti-HIV immune responses, beyond their utility as mere diagnostic tools.

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Legends for the Figures

Figure 1.

Distribution of patients's frequency regarding to the observed sensor activation promoted by individual sera (%). White bars represent the total number of patients, right-dashed bars non-treated patients and rhombus-filled bars the total of patients who had followed an ART.

Figure 2.

Scatter plots and correlation values between activation of the sensor and IgM (A) and IgG4 (B) titres.

Table 1. Clinical parameters and ART status of sera source patients.

Sera reference	Viral load (HIV-1 RNA copies)/ml [b]	$CD4^+$ cells/µl [c]	$CD8^+$ cells/µl [c]	ART [c]
Non-treated				
1	752855	10	150	
2	22500	300	612	
3	195000	616	1380	
4	167000	273	682	
5	28406	1049	935	
6	327165	388	957	
7	34328	273	na	
8	51028	610	1196	
9	20200	752	1040	
10	4270	572	1360	
11	72800	508	1680	
12	1960	584	1540	
13	5440	515	1470	
14	50800	276	959	
15	1520	240	1302	
16	71300	420	800	
17	150000	759	1182	
18	63856	1100	1952	
Treated				
Continuous treatment				
19	467433	10	1708	AZT,3TC,DDC
20	271648	180	1740	AZT, 3T, DDC
21	41063	285	2345	AZT, DDC
22	9564	350	622	DDI, HU
23	233430	46	472	3TC, D4T, SQV
24	53967	108	720	HU, D4T
25	57	770	1155	D4T, 3TC, IDV
26	<20	635	1271	ABV, 3TC, IDV
27	<20	515	528	ABV, 3TC, IDV
28	<20	884	nd	ABV, 3TC, IDV
29	<20	625	878	3TC ,NFV ,AZT
30	<20	631	711	DDI ,3TC ,NVP
31	<20	889	2148	ABV ,3TC ,NVP
32	<20	562	777	D4T ,IDV ,3TC
33	<20	392	595	DDI ,3TC ,NVP
34	<20	388	949	DDI ,3TC ,NVP
35	<20	1165	915	D4T ,3TC ,NFV
36	<20	944	1285	D4T ,3TC ,NFV
37	<20	342	935	D4T ,IDV ,3TC
38	<20	1236	1169	DDI ,IDV ,NFV ,NVP
Interrupted treatment				
39	15100	840	1610	DDI, D4T, 3TC
40	58500	352	1230	AZT, 3TC, ABC, EFV

41	113000	493	748	IDV, 3TC, AZT
42	14400	720	881	EFV, D4T, 3TC
43	15500	725	1485	ABV, DDI, LPV, RTV
44	12200	216	798	ABV, DDI, EFV
45	18900	342	1240	ABV, DDI, NVP
46	5130	405	822	EFV ,3TC ,AZT
47	19600	270	562	3TC, LPV, RTV, TDF
48	8630	840	865	ABV, 3TC, NVP
49	16700	342	1471	DDI, 3TC, NVP, TDF
50	9050	516	780	D4T, 3TC, NFV
51	6810	425	960	3TC, NFV, AZT
52	18400	1209	1211	ABV, 3TC, NVP
53	11700	902	1476	DDI, D4T, NFV
54	14765	814	910	D4T, 3TC, NFV
55	16100	756	1392	ABV ,3TC ,LPV ,RTV
56	6560	380	548	DDI, 3TC, LPV, RTV
57	15700	1287	1986	3TC ,NFV ,AZT

[a] Plasma HIV-1 RNA levels were measured by use of the ultrasensitive Amplicor monitor assay (Roche).

[b] CD4 and CD8 cell count was determined by flow cytometry.

[c] 3TC (Lamivudine), ABV (Abacavir), AZT (Zidovudine), D4T (Stavudine), DDI (Didanosine), EFV (Efavirenz), IDV (Indinavir), LPV (Lopinavir), NFV (Nelfinavir), NVP (Nevirapine), RTV (Ritonavir, PI), TDF (Tenofovir). na; data not available.

Table 2.

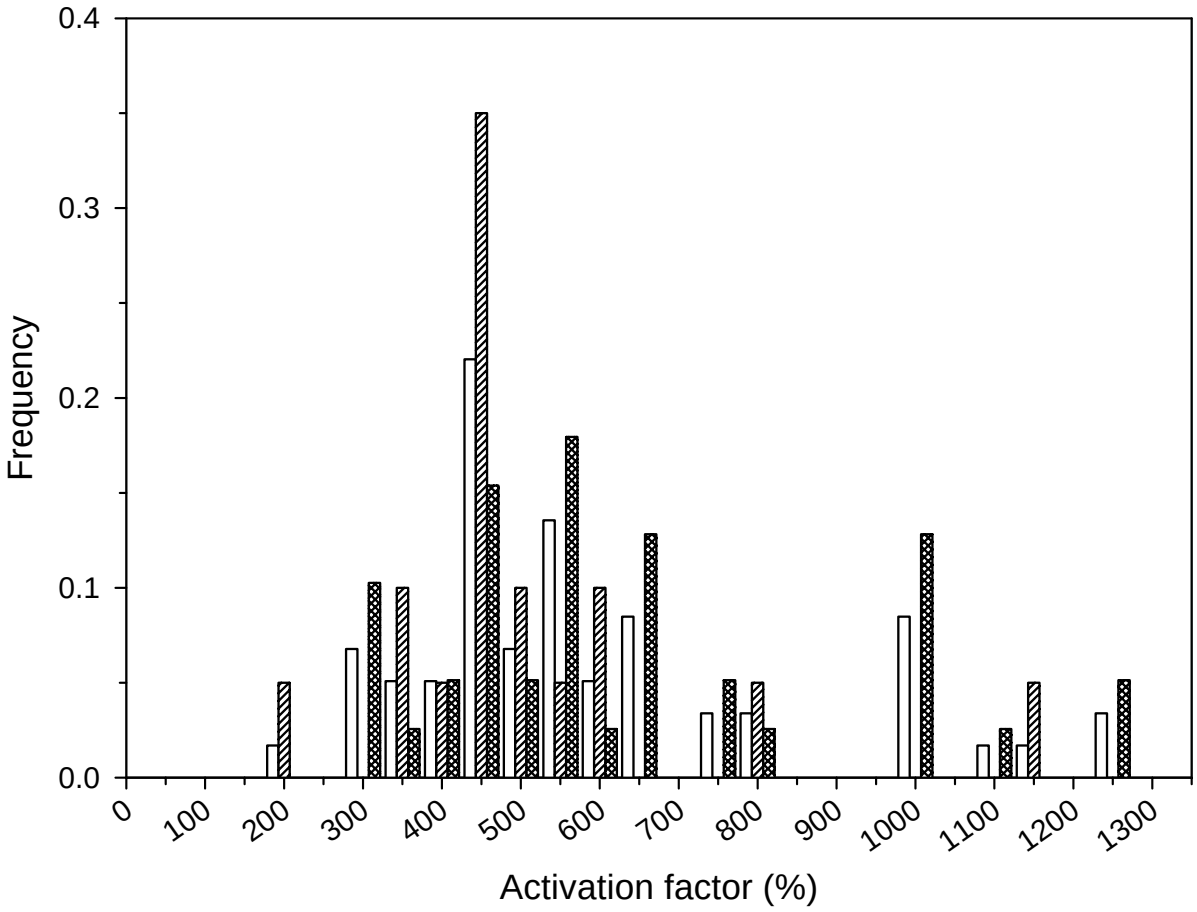
Significance of correlations (given as *p* values) between the sensor activation and the prevalence of immunoglobulin types in each serum.

Antibody	Non-treated	Treated	Total
IgG1	0.159	0.545	0.162
IgG2	0.946	0.811	0.874
IgG3	0.741	0.838	0.800
IgG4	0.128	0.098	0.025 *
IgA	0.803	0.528	0.533
IgE	0.099	0.364	0.105
IgM	0.005 *	0.671	0.201
VL[a]	0.642	0.388	0.140
CD4	0.506	0.535	0.305
CD8	0.057	0.812	0.592

* The asterisks indicate *p* values below 0.05.

[a] Relevant clinical parameters of patients source of sera have been included as controls.

Figure 1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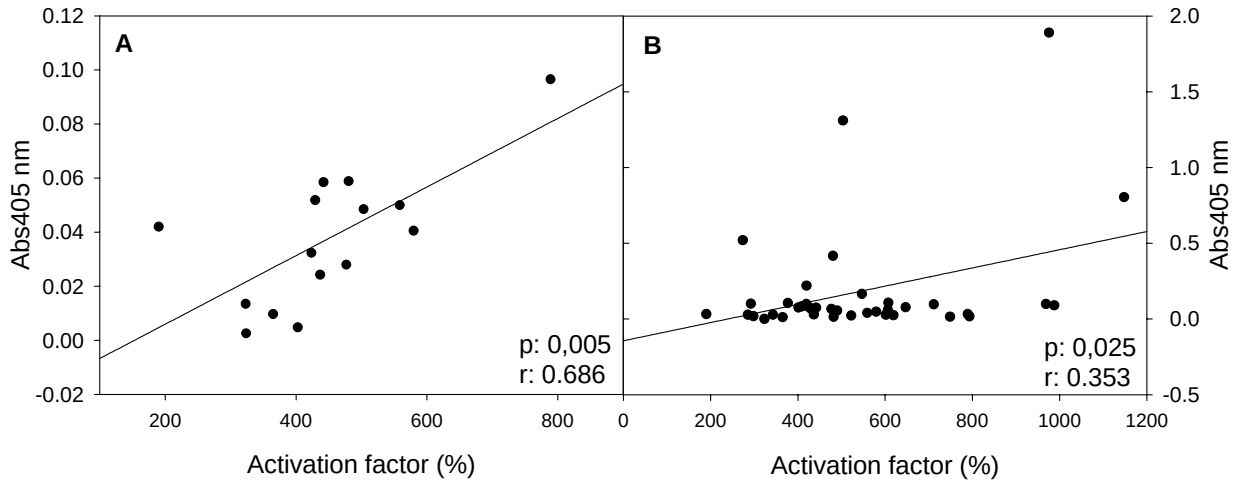
0.3
0.2
0.1
0.0
Frequency
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
Activation factor (%)

Figure 2


A
B
Abs405 nm
Abs405 nm
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
2.0
1.5
1.0
0.5
0.0
-0.5
200
400
600
800
0
200
400
600
800
1000
1200
p: 0,005
r: 0.686
p: 0,025
r: 0.353
Activation factor (%)
Activation factor (%)

References

1. Wei, X. *et al.* Antibody neutralization and escape by HIV-1. *Nature* **422**, 307-312 (2003).

2. Poignard, P., Klasse, P. J. & Sattentau, Q. J. Antibody neutralization of HIV-1. *Immunol. Today* **17**, 239-246 (1996).

3. Kwong, P. D. *et al.* HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* **420**, 678-682 (2002).

4. de Rosny, E., Vassell, R., Jiang, S., Kunert, R. & Weiss, C. D. Binding of the 2F5 monoclonal antibody to native and fusion-intermediate forms of human immunodeficiency virus type 1 gp41: implications for fusion-inducing conformational changes. *J. Virol.* **78**, 2627-2631 (2004).

5. Myszka, D. G. *et al.* Energetics of the HIV gp120-CD4 binding reaction. *Proc. Natl. Acad. Sci. U. S. A* **97**, 9026-9031 (2000).

6. Dhillon, A. K. *et al.* Dissecting the neutralizing antibody specificities of broadly neutralizing sera from human immunodeficiency virus type 1-infected donors. *J. Virol.* **81**, 6548-6562 (2007).

7. Ferrer-Miralles, N. *et al.* Engineering regulable Escherichia coli beta-galactosidases as biosensors for anti-HIV antibody detection in human sera. *J Biol Chem* **276**, 40087-40095 (2001).

8. Ferraz, R. M., Vera, A., Aris, A. & Villaverde, A. Insertional protein engineering for analytical molecular sensing. *Microb. Cell Fact.* **5**, 15 (2006).

9. Ferraz, R. M., Aris, A., Martinez, M. A. & Villaverde, A. High-throughput, functional screening of the anti-HIV-1 humoral response by an enzymatic nanosensor. *Mol. Immunol.* **43**, 2119-2123 (2006).

10. Aladid-Peralta, L. *et al.* Impact of highly active antiretroviral therapy on the maturation of anti-HIV-1 antibodies during primary HIV-1 infection. *HIV. Med.* **7**, 514-519 (2006).

11. Voltersvik, P. *et al.* Changes in immunoglobulin isotypes and immunoglobulin G (IgG) subclasses during highly active antiretroviral therapy: anti-p24 IgG1 closely parallels the biphasic decline in plasma viremia. *J. Acquir. Immune. Defic. Syndr.* **34**, 358-367 (2003).

12. Miller, J. H. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. (1972).

13. Sambrook, J., Fritsch, E. & Maniatis, T. *Molecular Cloning, A Laboratory Manual,* . Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

14. Binley, J. M. *et al.* The effect of highly active antiretroviral therapy on binding and neutralizing antibody responses to human immunodeficiency virus type 1 infection. *J. Infect. Dis.* **182**, 945-949 (2000).

15. Pellegrino, M. G. *et al.* HIV type 1-specific IgE in serum of long-term surviving children inhibits HIV type 1 production in vitro. *AIDS Res. Hum. Retroviruses* **18**, 363-372 (2002).

16. Fletcher, M. *et al.* Diagnosis of human immunodeficiency virus infection using an immunoglobulin E-based assay. *Clin. Diagn. Lab Immunol.* **7**, 55-57 (2000).

6.3. **ANNEX III**

III.A. INSERTIONAL PROTEIN ENGINEERING FOR ANALYTICAL MOLECULAR SENSING

Rosa María Ferraz, Andrea Vera, Anna Arís and Antonio Villaverde

Microbial Cell Factories 2006, 5:15

III.B. AGGREGATION AS BACTERIAL INCLUSION BODIES DOES NOT IMPLY INACTIVATION OF ENZYMES AND FLUORESCENT PROTEINS

Elena García-Fruitós, Nuria González-Montalbán, Montse Morell, Andrea Vera, Rosa María Ferraz, Anna Arís, Salvador Ventura and Antonio Villaverde

Microbial Cell Factories 2005, 4:27

6.3.1. ANNEX III.A

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Review

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Insertional protein engineering for analytical molecular sensing

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Abstract

The quantitative detection of low analyte concentrations in complex samples is becoming an urgent need in biomedical, food and environmental fields. Biosensors, being hybrid devices composed by a biological receptor and a signal transducer, represent valuable alternatives to non biological analytical instruments because of the high specificity of the biomolecular recognition. The vast range of existing protein ligands enable those macromolecules to be used as efficient receptors to cover a diversity of applications. In addition, appropriate protein engineering approaches enable further improvement of the receptor functioning such as enhancing affinity or specificity in the ligand binding. Recently, several protein-only sensors are being developed, in which either both the receptor and signal transducer are parts of the same protein, or that use the whole cell where the protein is produced as transducer. In both cases, as no further chemical coupling is required, the production process is very convenient. However, protein platforms, being rather rigid, restrict the proper signal transduction that necessarily occurs through ligand-induced conformational changes. In this context, insertional protein engineering offers the possibility to develop new devices, efficiently responding to ligand interaction by dramatic conformational changes, in which the specificity and magnitude of the sensing response can be adjusted up to a convenient level for specific analyte species. In this report we will discuss the major engineering approaches taken for the designing of such instruments as well as the relevant examples of resulting protein-only biosensors.

Review

Introduction

Conventional biosensors are hybrid elements consisting of a biochemical receptor for a given analyte, physically coupled to a physicochemical transducer that converts such interaction into a macroscopic, analytically useful signal [1]. In the last decades, many types of biosensors have been under continuous development, integrating biological components such as proteins, nucleic acids, membranes cells and even tissues acting as receptors, and different signal transducers devices including microbalances, electrodes, optical components and semiconductors. Such instruments have been applied into a diversity of fields but specially for the detection of contaminants in foods and environment [2]. More recently, and pressured by the need of more sensitive and specific detection tools for biomedical applications, in particular diagnosis, new types of protein-only biosensors are being explored [3], that contain both the receptor and transducer elements in a single polypeptide chain. Alternatively, protein-only

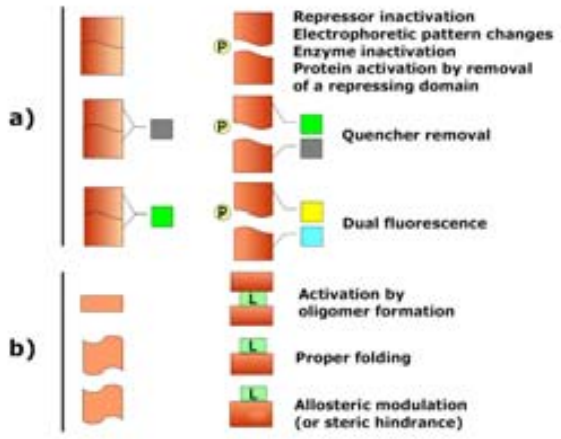

Figure 1
The biosensing principles of the constructs listed in Table 1 are summarized here as split in two groups. In **a)**, the sensing principles underlying cleavable platforms are presented in which simple hydrolysis of protease target site-bearing hybrid proteins by an effector protease (P) result in a macroscopic signal. Among others, variations of the migration pattern, enzyme activation or inactivation, repressor inactivation, enhanced fluorescence by removal of a quencher or dual fluorescence emission by FRET modulation. In **b)**, a ligand (L) promotes conformational modifications in the sensor either multimerization, correct folding or allosteric activation. A few enzyme biosensors are inactivated in presence of the ligand probably by steric hindrance of the active site.

sensors can specifically act as receptors that exploit the whole living cell where they are synthesised, as a complex signal transducer, enabling the detection of the analyte through intricate global activities such as differential growth or support of viral multiplication among others. Both type of protein-only biosensors offer very appealing advantages over classical devices. First, chemical coupling to the signal transducer is not required as straightforward bioproduction results in a ready-to-use final product, either a purified protein of protein-producing cells or cell extracts. Also, protein engineering procedures such as site-directed mutagenesis or directed molecular evolution allow refining the specificity of ligand binding and permit the development of new receptors for new analytes, as demanded from medicine or industry.

The mechanics for which a protein responds to a specific ligand in a macroscopically detectable way would generally imply variations in its activity, either enhancement or inhibition, that could be detectable either directly or indirectly through a biological amplification process. In general, natural protein-ligand interactions result in moderate conformational modifications that would be poorly useful in molecular switching, as they have a limited impact on protein's activity. Protein engineering allows the modification of the receptor in a way in which the interaction with the analyte promotes profound conformational modifications. There are many examples of useful intracellular indicators of molecular interactions, gene expression or for biological screening [4] that use at different extent end-to-end fusion proteins, including two-hybrid systems [5], fluorescence resonance energy transfer (FRET) [6], and protein fragment complementation [7] among others. However, insertional protein engineering allows a more versatile combination of functional modules for the construction of highly responsive mosaic proteins exhibiting unusual conformational versatility upon ligand binding [8,9]. Obviously, the protein segment or domain acting as a receptor element must be conveniently displayed on the protein surface to allow a proper interaction with the analyte. Although some of the constructs referenced below derive from random insertions and further selection [10,11], the previous identification of solvent-exposed permissive sites through different procedures has allowed a more rational designing procedures based on site directed peptide insertion for the construction of biosensors and other type of multifunctional proteins [12-19]. The principles of protein functionality supporting insertional approaches for biosensor construction are further discussed as exemplified by representative models and specific applications, being most of the resulting protein-only biosensors based on either cleavable (Figure 1A) or allosteric (Figure 1B) protein platforms. Representative examples of specific sensors and construction approaches are listed in Table 1.

Cleavable platforms

The most dramatic conformational modification that a given ligand (in this case a protease) might induce on a target protein is hydrolysis, that mostly result in its functional inactivation but being sometimes a requisite for a polypeptide reaching the active form, if existing as an inactive precursor. In fact, targeted proteolysis is a biological principle regulating many complex cellular events [20-22]. Therefore, including a specific protease target site on a protein's surface would made it susceptible to site-limited digestion resulting in detectable changes in its electrophoretic pattern. The successful implementation of such technology would imply a refined analysis of the protease target site susceptibility, as peptide display in different solvent-exposed sites could result in distinguishable digestion efficiencies, since the protein regions neighbouring the insert seem to have a dramatic influence on the peptide conformation [23]. This has been exemplified by the insertional mutagenesis of the protease resistant, green fluorescent protein (GFP), to make it susceptible to trypsin and other proteolytic enzymes [24]. The detection of specific proteases and proteolytic activities is now of extreme relevance in virology and in partic-

Table 1: Representative examples of protein only biosensors obtained by insertional mutagenesis.

Holding protein	Strategy	Insert	Analyte	Sensing mechanism	Signal (factor, when activated)	Application (proved or suggested)	References
β-galactosidase	Site directed insertion	FMDV[a] and HIV antigenic peptides	Anti-peptide antibodies and immune sera	Allosteric	Enzymatic activity up-shift (up to 12-fold)	Diagnosis	[38,39,43,47,48,49,59]
β-galactosidase	Site directed insertion	HIV protease substrate	HIV protease	Cleavage mediated inactivation	Enzymatic activity down-shift or electrophoretic analysis	Antiviral drug design and screening	[25,26]
Alkaline phosphatase	Site directed insertion	HIV antigenic peptide	Anti-peptide antibodies	Probably steric hindrance	Enzymatic activity down-shift	Diagnosis	[46]
Alkaline phosphatase	Site directed insertion plus site directed mutagenesis of the active site	HIV and HCV antigenic peptide	Anti-peptide antibodies	Allosteric	Enzymatic activity up-shift (up to 2.5-fold)	Diagnosis	[40]
GFP	Site directed insertion followed by random mutagenesis	TEM1 β-lactamase	TEM1 β-lactamase inhibitor	Allosteric	Fluorescence emission up-shift (not determined)	Drug design and screening	[41,42]
EGFP	Amino acid replacement	LPS/LA-binding motif	Bacterial LPS	Quenching	Fluorescence emission down-shift	Quality control (endotoxin detection)	[60]
TEM β-lactamase	Random insertion and phage-mediated selection	Random peptides	Anti PSA antibodies	Allosteric and steric hindrance upon the specific construct	Enzymatic activity down- or up-shift (up to 1.7-fold)	Diagnosis	[10]
p53	Site directed insertion plus site directed deletion	LF, HA and HSV antigenic peptides	Anti-peptide antibodies	Dimerization	Electrophoretic mobility up-shift (up to 100-fold)	Diagnosis and screening	[28]
p53	Site directed insertion	HIV and LF protease substrates	HIV protease and LF	Auto-inhibitory domain removal	Electrophoretic mobility up-shift (up to > 100-fold) or in situ hybridisation (2-fold)	Screening	[28]
cI lambda repressor	Site directed insertion	HIV, HCV and SARS protease substrates	HIV, HCV and SARS proteases	Cleavage mediated inactivation	Phage plaques counting (up to 50-fold)	Antiviral drug design and screening	[32,33,61]
MBP	Site directed insertion eventually followed by punctual mutagenesis	Zinc binding sites	Zinc	Allosteric	Fluorescence emission modulation (up to 8-fold)	Not specified, presumably wide	[62]
MBP	Random insertion	TEM-1 beta-lactamase segment	Maltose and other sugars	Allosteric	Enzymatic activity up-shift (up to 1.7-fold)	Not specified, presumably wide	[11]
DHFR	Site directed insertion eventually followed by punctual mutagenesis	FKBP macrolide-binding protein and ERα ligand binding domain	FK506 and estrogen	Binding-promoted thermostability and consequent genetic complementation	Growth of temperature-sensitive yeast under non-permissive temperatures (up to 2.5-fold)	Drug design and screening	[56]
FynSH3 [b]	Deletion	none	Proline-rich peptide ligand	Ligand induced protein folding	Tryptophan fluorescence increase (up to 15-fold)	Not specified, presumably wide	[55]
GFP-DsRed fusion [b]	Modular fusion	TEV protease substrate	TEV protease	Cleavage mediated fluorescent tag separation	Dual fluorescent emission yield	Antiviral drug design and screening	[29]

[a] Abbreviations are explained in the abbreviation list.
[b] A few examples of protein sensors obtained by either deletion or end-to-end fusion approaches are also shown.

ular for designing antiviral drugs that inhibit viral protein processing and therefore multiplication. Beyond the straightforward electrophoretic analysis of the sensing protein [25], a rather inconvenient technique from the analytical point of view, monitoring protease-mediated reduction of activity (fluorescence emission or enzymatic activity) would offer a more convenient protease sensing signal [26]. In a step further, it is known that many natural proteins are proteolytically activated by the removal of self inhibitory protein domains [27]. In this context, the convenient insertional engineering of the carboxy terminus of p53, containing such a regulatory element, has resulted in a set of p53 variants that are activated upon its removal mediated by either the lethal factor (LF) or the human immunodeficiency virus (HIV) protease [28]. Again, in this case, the sensing signal is detectable by upshift electrophoretic analysis, since the activated p53 gains the ability to interact with specific DNA sequences [28].

In an attempt to produce more convenient analytical signals, protease target sites have been introduced in the linker between two end-to-end fused proteins that emit fluorescence at different wavelengths, so the cleavage can be monitored by variations in the FRET spectra [29-31]. Although being not a standard insertional approach, the principles governing such engineering processes are similar to those discussed above. In this context, the protein hydrolysis splitting a fluorophore and its quencher has been also a successfully proven biosensing principle [60].

It would be expected that the generation of a signal by a specific proteolytic attack acted as an all-or-nothing switcher rather than as a fine sensing tool. However, a very discriminative monitoring tool for viral proteases activity was implemented as a high-throughput analytical method for antiviral drug testing and evaluation of the enzyme activity. The cI lytic repressor of the *E. coli* lambda bacteriophage has been engineered to accommodate a selected target site for proteases from either HIV [32], hepatitis C virus (HCV) [33] and severe acute respiratory syndrome (SARS) viruses [34]. The appropriate co-expression of the engineered cI and the protease promotes lytic lambda propagation that is reported by plaque counting. This system serves not only to test protease inhibitors for antiviral drug research but also to quantitatively evaluate the activity of proteases from mutant viruses emerging in patients treated with antiviral, protease-targeted drugs [35]. The cascade events supported by the cell as a network signal transducer permits the quantitative translation of the statistic cI hydrolysis within the cellular pool, what would be probably not possible by using a more straightforward signal transducing system.

Allosteric platforms

The regulatable activity of allosteric enzymes lies on a biological principle highly matching with the protein-only biosensing concept [36]. The activity of allosteric enzymes is modulated upon binding of an effector to a receptor site, that being different from the active site, can influence its performance through the conformational impact promoted by the allosteric effector. Since most natural effectors are irrelevant for analytical purposes, both allosteric and non allosteric enzymes have been engineered to allosterically respond to new effectors by insertion of appropriate receptor sites, in some cases accompanied by directed or random mutagenesis of the enzyme or directed molecular evolution. This straightforward insertional strategy often requires the identification of permissive sites in which inserted motives do not disturb irreversibly the enzyme activity [12,37], and has proven to be efficient in the engineering of β-galactosidase [38,39], alkaline phosphatase [40], β-lactamase [10] and GFP [41,42] as allosteric biosensors. As the fine mechanics of the conformational signal transduction in allosteric activation is not know, such devices have been constructed by error-and-trial approaches. Recently [11], a random insertional approach has permitted to newly create two allosteric enzymes by domain incorporation, by a strategy, in principle, with general applicability in biosensor design. Among enzyme inhibitors and other few ligand species that activate allosteric biosensors, antibodies have been noted to be specially efficient allosteric effectors [36] and the use of antigenic peptides as receptors in only-protein biosensors would offer appealing tools for the fast molecular diagnosis of infectious diseases [39,43]. Allosteric β-galactosidases displaying arginine-glycine-aspartic acid (RGD)-containing antigenic peptides [23], are activated by anti-peptide antibodies [38] but not by RGD-targeted integrins binding the same receptor [44]. This fact indicates different conformational constraints in the binding of both molecules [45] and suggests that the adaptive antibody binding could be a major force in sensor activation.

A main problem of allosteric biosensing is the poor signal-background ratio, that in most of cases does not reach more than 2-fold (Table 1). Higher activation factors would be extremely desirable for fine analytical applications where a wide dynamic range is required. In fact, in a few examples, the presence of anti-peptide antibodies (the analyte) even reduces the activity of the enzyme probably by steric hindrance of the active site, as reported by alkaline phosphatase [46] or β-lactamase [10], when the antigenic peptide was placed in the very close vicinity of the active site. The inhibition of the enzymatic activity is not very desirable as an analytical signal since in high-throughput analysis of complex samples, the presence of enzyme inhibitors might render false positives. By comparing different species of allosteric biosensors sensing

anti-peptide antibodies, it was recognized that the activation factor was highly depending on the perturbation that the inserted peptide receptor had promoted on the activity of the enzyme platform after insertion [47]. Greater was the reduction in the specific activity of the enzyme, higher the activation mediated by the effector, but reaching only activation factors around 2 that seemed to be a biological upper limit to allosteric activation [47]. However, a deeper exploration of β-galactosidase allosteric sensors has revealed that the signal background ratio can be enhanced up to more than 10-fold, by alternative or combined approaches such as introducing a higher number of receptors per enzyme [43,48], optimising the reaction conditions [49] and selecting the appropriate substrate [50].

Apart from plain diagnostic utilities, allosteric sensors can intriguingly perform as tools for the analysis of the immune response, as they specially recognize antibodies with a high potential as modifiers of the epitope conformation. In this context, a β-galactosidase sensor displaying an HIV gp41 epitope and responsive to human HIV-immune sera is preferentially activated by the IgG4 antibody subpopulation [51]. As at least in the case of HIV infection the ability of anti-viral antibodies to modify the epitope's conformation is strongly related to their neutralizing activity [52,53] and probably to the progression of the infection [54], allosteric biosensing could eventually offer a valuable instrument for high-throughput sera analysis for prognostic investigation.

Other examples of conformation-dependent sensor activation

Conformational changes promoted by molecular interactions may generate signals suitable for biosensing other than allosteric responses. The correct folding of a deletion mutant of the human Fyn tyrosine kinase (FynSH3), a predominantly β-sheet protein, is induced by the binding of an appropriate proline-rich peptide ligand, and the folding process monitored in real time by tryptophan fluorescence [55]. Temperature-sensitive yeast cells lacking dihydropholate reductase (DHFR) are complemented by two mouse DHFR containing foreign different ligand binding domains [56]. Culture growth is then enhanced in presence of the respective ligands proving that molecular binding activates the complementing enzyme. Although this system can be observed as a generic biosensor [57], its real potential would lie on the selection of specific or improved ligands by directed molecular-cellular evolution. On the other hand, the presence of bivalent antibodies can promote dimer formation of a mutant p53 in which the tetramerization domain has been removed and antigenic B-cell epitopes of viral origin conveniently inserted [28]. Since dimers are much more active than monomers, the presence of antiviral antibodies enables p53 to bind DNA in an electrophoretically detectable manner.

Other conformation-linked effects of molecular interactions might also result in detectable activity changes or phenotypes acting as macroscopic signals for a given analyte. Gaining further knowledge about enzyme structure and dynamics would necessarily offer additional possibilities of rational protein engineering [58] for exploitation of such conformational signals.

Conclusion

Insertion of foreign peptides as receptors of protein-only biosensors confers the resulting protein construct the ability to sense analytes by dramatic conformational changes unusual in the native, non engineered protein. For such a sensor being efficiently responsive, appropriated permissive sites need to be selected permitting proper receptor display and signal transduction, and the whole protein might require further engineering to gain specificity and response range. Although most protein-only biosensors derive from trial-and-error engineering approaches, rational and very clever setting-ups are exemplified by combinations of sensing protein segments and conveniently modified acceptor proteins. Among the diversity of sensing strategies based on insertional mutagenesis two protein platforms emerge as the most explored, namely cleavable sensors responding to proteases or their inhibitors, and allosteric, among whose most efficient effectors are antibodies. The performance of these two sensor types has been largely proved in the high throughput screening of antiviral drugs and for the molecular diagnosis of infectious diseases respectively. Although the potential applications of protein-only biosensors are diverse and still have to be fully exploited, they have arisen as valuable new tools in biomedicine being intriguing alternatives to classical sensing technologies.

Authors' contributions

Rosa M Ferraz and Andrea Vera have equally contributed to this review.

Abbreviations

DHFR Dihydropholate reductase

DsRed Engineered mutant of red fluorescent protein

EGFP Enhanced green fluorescent protein

FMDV Food-and-mouth disease virus

FRET Fluorescence resonance energy transfer

GFP Green fluorescent protein

HA Influenza hemaglutinin

HCV Hepatitis C virus

HIV Human immunodeficiency virus

HSV Herpes simplex virus

LA Lipid A

LF Lethal factor

LPS Lipopolysaccharide

MBP Maltose binding protein

RGD Arginine-glycine-aspartic acid tri-peptide

SARS Severe acute respiratory syndrome

TEV Tobacco etch virus

TEM β lactamase

PSA Prostate specific antigen

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References

1. Thevenot DR, Toth K, Durst RA, Wilson GS: **Electrochemical biosensors: recommended definitions and classification.** *Biosens Bioelectron* 2001, **16:**121-131.
2. Rich RL, Myszka DG: **Survey of the year 2004 commercial optical biosensor literature.** *J Mol Recognit* 2005, **18:**431-478.
3. Cooper MA: **Biosensor profiling of molecular interactions in pharmacology.** *Curr Opin Pharmacol* 2003, **3:**557-562.
4. Piehler J: **New methodologies for measuring protein interactions in vivo and in vitro.** *Curr Opin Struct Biol* 2005, **15:**4-14.
5. Toby GG, Golemis EA: **Using the yeast interaction trap and other two-hybrid-based approaches to study protein-protein interactions.** *Methods* 2001, **24:**201-217.
6. Yan Y, Marriott G: **Analysis of protein interactions using fluorescence technologies.** *Curr Opin Chem Biol* 2003, **7:**635-640.
7. Obrdlik P, El Bakkoury M, Hamacher T, Cappellaro C, Vilarino C, Fleischer C, Ellerbrok H, Kamuzinzi R, Ledent V, Blaudez D, Sanders D, Revuelta JL, Boles E, Andre B, Frommer WB: **K+ channel interactions detected by a genetic system optimized for systematic studies of membrane protein interactions.** *Proc Natl Acad Sci U S A* 2004, **101:**12242-12247.
8. Doi N, Yanagawa H: **Insertional gene fusion technology.** *FEBS Lett* 1999, **457:**1-4.
9. Ostermeier M: **Engineering allosteric protein switches by domain insertion.** *Protein Eng Des Sel* 2005, **18:**359-364.
10. Legendre D, Soumillion P, Fastrez J: **Engineering a regulatable enzyme for homogeneous immunoassays.** *Nat Biotechnol* 1999, **17:**67-72.
11. Guntas G, Ostermeier M: **Creation of an allosteric enzyme by domain insertion.** *J Mol Biol* 2004, **336:**263-273.
12. Feliu JX, Villaverde A: **Engineering of solvent-exposed loops in Escherichia coli beta-galactosidase.** *FEBS Lett* 1998, **434:**23-27.
13. Hiraga K, Yamagishi A, Oshima T: **Mapping of unit boundaries of a protein: exhaustive search for permissive sites for duplication by complementation analysis of random fragment libraries of tryptophan synthase alpha subunit.** *J Mol Biol* 2004, **335:**1093-1104.
14. Charbit A, Ronco J, Michel V, Werts C, Hofnung M: **Permissive sites and topology of an outer membrane protein with a reporter epitope.** *J Bacteriol* 1991, **173:**262-275.
15. Martineau P, Guillet JG, Leclerc C, Hofnung M: **Expression of heterologous peptides at two permissive sites of the MalE protein: antigenicity and immunogenicity of foreign B-cell and T-cell epitopes.** *Gene* 1992, **113:**35-46.
16. Manoil C, Bailey J: **A simple screen for permissive sites in proteins: analysis of Escherichia coli lac permease.** *J Mol Biol* 1997, **267:**250-263.
17. Coeffier E, Clement JM, Cussac V, Khodaei-Boorane N, Jehanno M, Rojas M, Dridi A, Latour M, El Habib R, Barre-Sinoussi F, Hofnung M, Leclerc C: **Antigenicity and immunogenicity of the HIV-1 gp41 epitope ELDKWA inserted into permissive sites of the MalE protein.** *Vaccine* 2000, **19:**684-693.
18. Aris A, Villaverde A: **Modular protein engineering for non-viral gene therapy.** *Trends Biotechnol* 2004, **22:**371-377.
19. Bckstrom M, Holmgren J, Schodel F, Lebens M: **Characterization of an internal permissive site in the cholera toxin B-subunit and insertion of epitopes from human immunodeficiency virus-1, hepatitis B virus and enterotoxigenic Escherichia coli.** *Gene* 1995, **165:**163-171.
20. Ehrmann M, Clausen T: **Proteolysis as a regulatory mechanism.** *Annu Rev Genet* 2004, **38:**709-724.
21. Goulet B, Nepveu A: **Complete and limited proteolysis in cell cycle progression.** *Cell Cycle* 2004, **3:**986-989.
22. Hilt W: **Targets of programmed destruction: a primer to regulatory proteolysis in yeast.** *Cell Mol Life Sci* 2004, **61:**1615-1632.
23. Benito A, Mateu MG, Villaverde A: **Improved mimicry of a foot-and-mouth disease virus antigenic site by a viral peptide displayed on beta-galactosidase surface.** *Biotechnology (N Y)* 1995, **13:**801-804.
24. Chiang CF, Okou DT, Griffin TB, Verret CR, Williams MN: **Green fluorescent protein rendered susceptible to proteolysis: positions for protease-sensitive insertions.** *Arch Biochem Biophys* 2001, **394:**229-235.
25. Baum EZ, Bebernitz GA, Gluzman Y: **beta-Galactosidase containing a human immunodeficiency virus protease cleavage site is cleaved and inactivated by human immunodeficiency virus protease.** *Proc Natl Acad Sci U S A* 1990, **87:**10023-10027.
26. Vera A, Aris A, Daura X, Martinez MA, Villaverde A: **Engineering the E. coli beta-galactosidase for the screening of antiviral protease inhibitors.** *Biochem Biophys Res Commun* 2005, **329:**453-456.
27. Pufall MA, Graves BJ: **Autoinhibitory domains: modular effectors of cellular regulation.** *Annu Rev Cell Dev Biol* 2002, **18:**421-462.
28. Geddie ML, O'Loughlin TL, Woods KK, Matsumura I: **Rational design of p53, an intrinsically unstructured protein, for the fabrication of novel molecular sensors.** *J Biol Chem* 2005, **280:**35641-35646.
29. Kohl T, Heinze KG, Kuhlemann R, Koltermann A, Schwille P: **A protease assay for two-photon crosscorrelation and FRET analysis based solely on fluorescent proteins.** *Proc Natl Acad Sci U S A* 2002, **99:**12161-12166.
30. Zhang B: **Design of FRET-based GFP probes for detection of protease inhibitors.** *Biochem Biophys Res Commun* 2004, **323:**674-678.
31. Nagai T, Miyawaki A: **A high-throughput method for development of FRET-based indicators for proteolysis.** *Biochem Biophys Res Commun* 2004, **319:**72-77.
32. Goh YY, Frecer V, Ho B, Ding JL: **Rational design of green fluorescent protein mutants as biosensor for bacterial endotoxin.** *Protein Eng* 2002, **15:**493-502.
33. Martinez MA, Cabana M, Parera M, Gutierrez A, Este JA, Clotet B: **A bacteriophage lambda-based genetic screen for characterization of the activity and phenotype of the human immunodeficiency virus type 1 protease.** *Antimicrob Agents Chemother* 2000, **44:**1132-1139.

34. Martinez MA, Clotet B: **Genetic screen for monitoring hepatitis C virus NS3 serine protease activity.** *Antimicrob Agents Chemother* 2003, **47:**1760-1765.
35. Parera M, Clotet B, Martinez MA: **Genetic screen for monitoring severe acute respiratory syndrome coronavirus 3C-like protease.** *J Virol* 2004, **78:**14057-14061.
36. Cabana M, Fernandez G, Parera M, Clotet B, Martinez MA: **Catalytic efficiency and phenotype of HIV-1 proteases encoding single critical resistance substitutions.** *Virology* 2002, **300:**71-78.
37. Villaverde A: **Allosteric enzymes as biosensors for molecular diagnosis.** *FEBS Lett* 2003, **554:**169-172.
38. Benito A, Villaverde A: **Insertion of a 27 amino acid viral peptide in different zones of Escherichia coli beta-galactosidase: effects on the enzyme activity.** *FEMS Microbiol Lett* 1994, **123:**107-112.
39. Benito A, Feliu JX, Villaverde A: **Beta-galactosidase enzymatic activity as a molecular probe to detect specific antibodies.** *J Biol Chem* 1996, **271:**21251-21256.
40. Ferrer-Miralles N, Feliu JX, Vandevuer S, Muller A, Cabrera-Crespo J, Ortmans I, Hoffmann F, Cazorla D, Rinas U, Prevost M, Villaverde A: **Engineering regulable Escherichia coli beta-galactosidases as biosensors for anti-HIV antibody detection in human sera.** *J Biol Chem* 2001, **276:**40087-40095.
41. Brennan CA, Christianson K, La Fleur MA, Mandecki W: **A molecular sensor system based on genetically engineered alkaline phosphatase.** *Proc Natl Acad Sci U S A* 1995, **92:**5783-5787.
42. Doi N, Yanagawa H: **Design of generic biosensors based on green fluorescent proteins with allosteric sites by directed evolution.** *FEBS Lett* 1999, **453:**305-307.
43. Doi N, Yanagawa H: **Evolutionary design of generic green fluorescent protein biosensors.** *Methods Mol Biol* 2002, **183:**49-55.
44. Feliu JX, Ferrer-Miralles N, Blanco E, Cazorla D, Sobrino F, Villaverde A: **Enhanced response to antibody binding in engineered beta-galactosidase enzymatic sensors.** *Biochim Biophys Acta* 2002, **1596:**212-224.
45. Alcala P, Feliu JX, Aris A, Villaverde A: **Efficient accommodation of recombinant, foot-and-mouth disease virus RGD peptides to cell-surface integrins.** *Biochem Biophys Res Commun* 2001, **285:**201-206.
46. Feliu JX, Benito A, Oliva B, Aviles FX, Villaverde A: **Conformational flexibility in a highly mobile protein loop of foot-and-mouth disease virus: distinct structural requirements for integrin and antibody binding.** *J Mol Biol* 1998, **283:**331-338.
47. Brennan C, Christianson K, Surowy T, Mandecki W: **Modulation of enzyme activity by antibody binding to an alkaline phosphatase-epitope hybrid protein.** *Protein Eng* 1994, **7:**509-514.
48. Ferrer-Miralles N, Feliu JX, Villaverde A: **Molecular mechanisms for antibody-mediated modulation of peptide-displaying enzyme sensors.** *Biochem Biophys Res Commun* 2000, **275:**360-364.
49. Cazorla D, Feliu JX, Ferrer-Miralles N, Villaverde A: **Tailoring molecular sensing for peptide displaying engineered enzymes.** *Biotechnology Letters* 2002, **24:**467-477.
50. Ferraz RM, Aris A, Villaverde A: **Profiling the allosteric response of an engineered beta-galactosidase to its effector, anti-HIV antibody.** *Biochem Biophys Res Commun* 2004, **314:**854-860.
51. Ferraz RM, Aris A, Villaverde A: **Enhanced molecular recognition signal in allosteric biosensing by proper substrate selection.** *Biotechnol Bioeng* 2006, **94(1):**193-9.
52. Ferraz RM, Aris A, Martinez MA, Villaverde A: **High-throughput, functional screening of the anti-HIV-1 humoral response by an enzymatic nanosensor.** *Mol Immunol* 2006, **43(13):**2119-23.
53. de Rosny E, Vassell R, Jiang S, Kunert R, Weiss CD: **Binding of the 2F5 monoclonal antibody to native and fusion-intermediate forms of human immunodeficiency virus type 1 gp41: implications for fusion-inducing conformational changes.** *J Virol* 2004, **78:**2627-2631.
54. Kwong PD, Doyle ML, Casper DJ, Cicala C, Leavitt SA, Majeed S, Steenbeke TD, Venturi M, Chaiken I, Fung M, Katinger H, Parren PW, Robinson J, Van Ryk D, Wang L, Burton DR, Freire E, Wyatt R, Sodroski J, Hendrickson WA, Arthos J: **HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites.** *Nature* 2002, **420:**678-682.
55. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM: **Antibody neutralization and escape by HIV-1.** *Nature* 2003, **422:**307-312.
56. Kohn JE, Plaxco KW: **Engineering a signal transduction mechanism for protein-based biosensors.** *Proc Natl Acad Sci U S A* 2005, **102:**10841-10845.
57. Tucker CL, Fields S: **A yeast sensor of ligand binding.** *Nat Biotechnol* 2001, **19:**1042-1046.
58. Vidan S, Snyder M: **Making drug addicts out of yeast.** *Nat Biotechnol* 2001, **19:**1022-1023.
59. Agarwal PK: **Enzymes: An integrated view of structure, dynamics and function.** *Microb Cell Fact* 2006, **5:**2.
60. Feliu JX, Ramirez E, Villaverde A: **Distinct mechanisms of antibody-mediated enzymatic reactivation in beta-galactosidase molecular sensors.** *FEBS Lett* 1998, **438:**267-271.
61. Marvin JS, Hellinga HW: **Conversion of a maltose receptor into a zinc biosensor by computational design.** *Proc Natl Acad Sci U S A* 2001, **98:**4955-4960.

6.3.2. ANNEX III.B

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Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins

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Abstract

Background: Many enzymes of industrial interest are not in the market since they are bio-produced as bacterial inclusion bodies, believed to be biologically inert aggregates of insoluble protein.

Results: By using two structurally and functionally different model enzymes and two fluorescent proteins we show that physiological aggregation in bacteria might only result in a moderate loss of biological activity and that inclusion bodies can be used in reaction mixtures for efficient catalysis.

Conclusion: This observation offers promising possibilities for the exploration of inclusion bodies as catalysts for industrial purposes, without any previous protein-refolding step.

Background

Protein misfolding is a common event during bacterial over-expression of recombinant genes [1]. The aggregation of insoluble polypeptide chains as inclusion bodies has seriously restricted the spectrum of proteins marketed by the biotechnology industry. Being widespreadly believed that inclusion body proteins are biologically inactive and therefore useless in bioprocesses, many aggregation-prone products have been disregarded for commercialisation. Protein solubility can be tailored by either process [2] or protein [3] engineering, although most efforts have been addressed to minimize inclusion body formation by co-production of folding modulators [4], or to refold purified inclusion body proteins by chemical denaturation followed by refolding procedures [5]. Both strategies need to be adapted to particular protein species and they render largely variable results regarding the final soluble protein yield.

Interestingly, independent reports have noted enzymatic activity associated to inclusion bodies formed by recombinant enzymes [6-8], but the extent of these side-observations has been never quantified and its biological and biotechnological relevance remained unexplored. In this work, we have quantitatively explored the biological

Table 1: Enzymatic activity or fluorescence of inclusion bodies produced in *E. coli*

Construct name	Reference	Functional protein	Fraction of inclusion body protein (range, %) [a]	Aggregating domain or protein (all in the N-terminal position)	Specific activity or emission [b] (enzymatic units/mg or fluorescence units/mg)		Activity of the inclusion body fraction relative to that of soluble protein (%) [c]
					Soluble protein	Inclusion bodies	
VP1LAC	This work and [9]	*E. coli* β-galactosidase	35.6–45.9	FMDV VP1 capsid protein	698.3 ± 153.0	1162.5 ± 256.0	166.4
hDHFR	[25]	Human dihydrofolate reductase	28.4–36.8	none	$8.0\ 10^{-2} \pm 2.6\ 10^{-2}$	$4.7\ 10^{-3} \pm 0.9\ 10^{-3}$	5.9
VP1GFP	This work	Green fluorescent protein	82.5–88.4	FMDV VP1 capsid protein	359.5 ± 66.0	70.4 ± 10.1	19.5
Aβ42(F19D)-BFP	[26]	Blue fluorescent protein	61.4–65.3	Aβ42(F19D)	118.1 ± 10.2	36.3 ± 2.2	30.7

[a] The percentage of protein found in inclusion bodies relative to the total intracellular amount of recombinant protein. Values were determined from different samples taken at 3 and 5 h after triggering recombinant gene expression.
[b] These values were determined in samples taken between 3 and 5 h after triggering recombinant gene expression.
[c] Specific activity or fluorescence emission of inclusion bodies relative to the values determined for the soluble counterpart fraction. Protein amounts were determined by Western blot analysis as described and enzymatic assays performed by conventional procedures. Excitation wavelengths were 450 nm for VP1GFP and 360 nm for Aβ42(F19D)-BFP.

activity of inclusion body recombinant proteins and their potential use for bioprocesses in the aggregated form.

Results

To determine the occurrence of active protein in inclusion bodies we analysed those formed upon overproduction of the wild-type human dihydrofolate reductase (hDHFR) and an engineered *E. coli* β-galactosidase fused to the aggregation-prone foot-and-mouth disease virus (FMDV) VP1 capsid protein (VP1LAC). In addition, we explored fluorescence emission of green and blue fluorescent proteins (GFP and BFP respectively) fused to different aggregating polypeptides, namely the FMDV VP1 and a point mutant of the human Aβ-amyloid peptide (Aβ(F19D)), by comparing specific fluorescence emission of protein in the soluble cell fraction and purified inclusion bodies. Upon overproduction, all these proteins form cytoplasmic inclusion bodies in *E. coli*, the fraction of the aggregated protein ranging between 28 and 88 % of the total recombinant production (Table 1). Surprisingly, both enzymatic activity and specific fluorescence of inclusion body proteins were unexpectedly high (Table 1), ranging from 6 to 166 % of that of their counterparts occurring in the soluble cell fraction. This fact indicates that protein inactivation mediated by *in vivo* aggregation is only moderate. In addition, it is shown that protein packaging as bacterial inclusion bodies into inter-molecular β-sheet architecture (characterized by the presence of a peak around 1620 cm^{-1} that dominates the FTIR spectrum in the amide I region) [9,10] in these model proteins (Figure 1) is compatible with the functionality of enzyme active sites and fluorophores. In this context, VP1GFP and Aβ42(F19D)-BFP inclusion bodies are noticeably fluorescent inside the producing cells (Figure 2).

We wondered if active inclusion bodies could be then used in suspension as efficient catalysts for bioprocesses. If so, the straightforward use of these particles, that in addition are easily removable from the reaction mixture once the reaction is completed by low speed centrifugation, would be a convenient alternative to in vitro protein refolding before use, a complex procedure for which efficiencies are highly variable but in general low [5]. The enzymatic activity of soluble and inclusion body versions of both VP1LAC and hDHFR was then monitored in reaction mixtures. As observed (Figure 3A and 3B), inclusion body-embedded enzymes performed very efficiently as catalysts of enzymatic reactions. Substrate hydrolysis mediated by the insoluble form of VP1LAC was significantly faster than that mediated by the same amount of the soluble version (Figure 3A), while substrate processing by hDHFR was slower when driven from inclusion bodies but still important (Figure 3B). These observations are nicely compatible with the specific activities displayed by both versions of these proteins (Table 1).

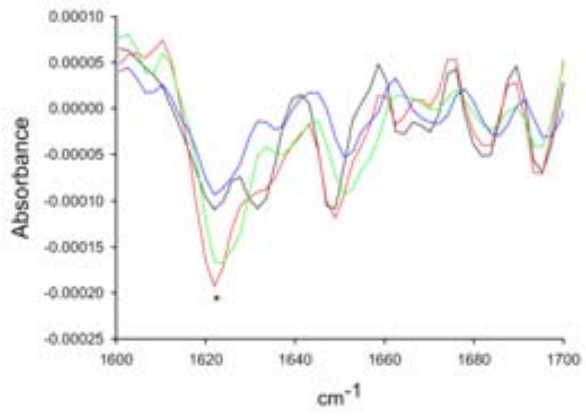

Figure 1
FTIR spectra of inclusion bodies formed by either VP1LAC (black), hDHFR (green), VP1GFP (red) or Aβ42(F19D)-BFP (blue) in the amide I region [9]. The asterisk labels the peak indicative of extended inter-molecular β-sheet structures in bacterial inclusion bodies.

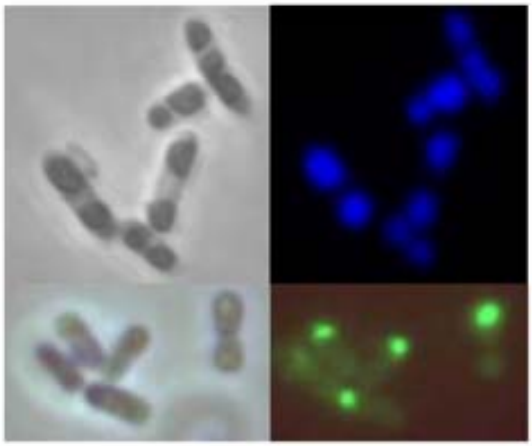

Figure 2
Optical micrographs of Aβ42(F19D)-BFP (top) and VP1GFP (bottom) inclusion bodies by phase contrast (left) and fluorescent microscopy (right).

Discussion

The quantitative similarity between protein activity in the soluble cell fraction and that of the aggregated forms of both enzymes and fluorescent proteins (Table 1) demonstrates that physiological aggregation as inclusion bodies does not necessarily split protein population into active and inactive fractions. Probably, protein solubility (observed as the occurrence in the soluble cell fraction) does not necessarily indicate the acquisition of a correctly folded and thus active structure. In this context, soluble micro-aggregates have been described [11] and recently characterized in detail [12]. The non complete coincidence between solubility and folding has been previously indicated by exhaustive mutational analysis of model proteins [13], showing that the genetic determinants of protein aggregation and misfolding are not coincident. In this way, natively unfolded proteins are unstructured but soluble [14]. Therefore, determinations of GFP-fusions solubility by using fluorescence as reporter [15] could have eventually been indicative of folding-misfolding extend rather than solubility-insolubility, since inclusion bodies formed by GFP fusions can be highly fluorescent (Figure 2). Furthermore, solubility does not appear to be an all-or-nothing attribute and polypeptide chains might exhibit a continuum of folding states in both soluble and insoluble cell fractions, between which they are dynamically transferred with the assistance of cellular folding modulators [16]. In this context, the occurrence and evolution of 'soluble' aggregates in bacteria (namely misfolded species occurring in the soluble cell fraction and presumably inactive) [12] could explain the variable specific activity observed in the soluble cell fraction of bacteria producing recombinant β-galactosidases [17].

Inversely, our results prove a major occurrence of native or native-like protein in inclusion bodies. In fact, deposition as inclusion bodies might even result in the enrichment of active species as suggested by the specific activity (166 % of that found in the soluble cell fraction; Table 1) and catalytic properties (Figure 3A) of VP1LAC inclusion bodies. This observation can be then again indirectly indicative of the presence of enzymatically inactive protein in the soluble cell fraction, since protein deposition is not expected to favour a correct folding.

Finally, although the existence of native-like structure in bacterial inclusion body proteins has been previously reported [18], here we demonstrate that this is not anecdotic but probably the architectonic nature of these kind of aggregates, as inclusion bodies formed by four structurally different proteins all display significantly high biological activity. Interestingly, the active and properly folded polypeptides in inclusion bodies coexist with a molecular β-sheet organization also manifest in all cases, although the extent of β-sheet structure and its coincidence with the biological activity of the aggregates cannot be quantitatively evaluated. Since is highly improbable that enzyme active sites involved in the intermolecular β-sheet struc-

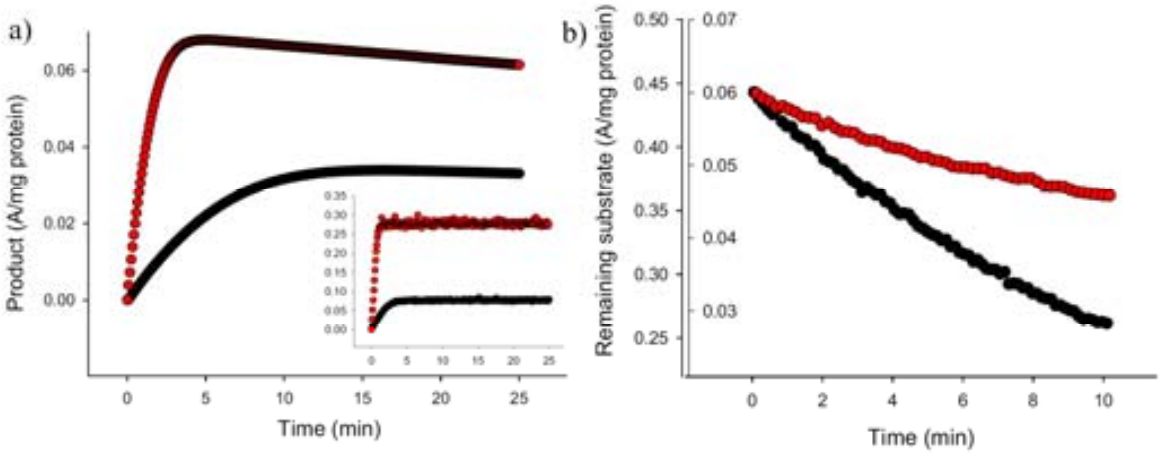

Figure 3
A) Product formed by soluble (black symbols) or inclusion body (red symbols) VP1LAC through ONPG hydrolysis as determined at 414 nm. Very coincident results have been obtained by using CPRG as alternative substrate (see the small panel), whose hydrolysis product was determined at 540 nm. B) Conversion of NADPH into $NADP^+$ associated to tetrahydrofolate formation mediated by soluble (black symbols, left scale) and inclusion body (red symbols, right scale) hDHFR. Absorbance was determined at 340 nm.

ture could be themselves active, we suggest that enzymatic activity or fluorescence are supported by properly folded molecules or molecule segments. Aggregation, observed as protein deposition driven by intermolecular interactions between solvent-exposed hydrophobic patches [9] would not necessarily disturb the conformation of all protein domains, and the active site would be still functional if misfolded, aggregation-prone regions are located in a distant site of the polypeptide chain. Alternatively, properly folded and active molecules could coexist with β-sheet-enriched (inactive) versions of the same species, and both situations could in fact take place simultaneously in single aggregate units. Further structural and functional analysis would hopefully solve this issue.

From an applied point of view, inclusion bodies, being formed by sequence-specific interaction between homologous protein patches result in highly pure protein microparticles [9]. Since they are also porous and highly hydrated [19], efficient substrate diffusion would probably occur for most of the (or at least many) biotechnologically relevant aggregated enzymes, thus opening the possibility for a new industrial market of enzymatically active inclusion bodies.

Conclusion

Results presented here prove that aggregation of recombinant proteins as bacterial inclusion bodies does not necessarily inactivate them, despite the enriched intermolecular β-sheet structure observed in those formed by the tested model proteins. The extent of protein activity varies depending on the specific protein, but even the lowest functional values observed are still high enough to consider the use of inclusion body enzymes in bioprocesses, without any previous refolding step. The eventual incorporation of inclusion bodies in industrial catalysis could represent an important conceptual shift in the biotechnology market.

Methods

Strain, plasmids and culture conditions

E. coli MC4100 [20] was used for all the experiments. Plasmids encoding hDHFR and Aβ42(F19D)-BFP have been previously described and appropriate references can be found in Table 1. Briefly, in the Aβ42(F19D)-BFP vector (6.7 Kb) the DNA sequence encoding the 42-mer Alzheimer's amyloid peptide, (bearing a Phe^{19}→Asp mutation to reduce its *in vivo* aggregation rate), is fused upstream of the BFP gene and under the control of the T7 promoter, in a pET-28 based vector. In the product, the two protein sequences were separated by 12-mer linker stretch to provide flexibility to the fusion protein and limit steric constraints between domains. pTVP1LAC was constructed by moving the *Sal*I-*Nco*I VP1LAC fusion-encoding DNA segment (3.5 Kb) from pJVP1LAC (8.5 Kb) to the cloning vector pTRC99A [20]. The resulting pTVP1LAC construct

(7.7 Kb) was used to direct the production of VP1LAC. The *lacZ* gene was further replaced there by an appropriate GFP-encoding DNA segment (0.7 Kb) through digestion with *Eco*RI and *Bam*HI, rendering pTVP1GFP (5.5 Kb). All the production processes were performed in shaker-flask cultures growing at 37°C in LB rich medium [20] plus 100 μg/ml ampicillin for plasmid maintenance, and recombinant gene expression was induced when the OD_{550} reached 0.4, by adding 1 mM IPTG. Cell samples were taken at 3 and 5 h after induction of gene expression.

Analysis of enzymatic activity

Culture samples of 2.5 ml were jacketed in ice, disrupted by sonication for 5 min at 50 W under 0.5 s cycles [21] and centrifuged at 4°C for 15 min at 15000 g. The supernatant was directly used for the analysis as the soluble cell fraction. Inclusion bodies were purified by a detergent-washing protocol as described [19] and used in suspension for activity analysis. β-Galactosidase activity of both soluble cell fraction and inclusion bodies of VP1LAC was determined in microplates as described [7,22] under continuous stirring at 250 rpm. Kinetic analysis of VP1LAC enzymatic activity was monitored in 120 μl reaction mixtures with either 2 mM ONPG (pH 8.4) or 2 mM CPRG (pH 7.0). The hDHFR activity was determined by incubating 50 μl of the protein sample and 850 μl of the appropriate assay buffer (0.1 M K_3PO_4 pH 7.4, 1 mM DTT, 0.5 M KCl, 1 mM EDTA and 20 mM ascorbic acid) for 10 minutes at room temperature. Then, 50 μl of 2 mM 7,8-dihidrofolate and 50 μl of 2 mM NADPH were added and hDHFR activity was recorded every 15 seconds during 4 minutes at 340 nm. Protein concentration in all the assays was adjusted between 2 and 3 μg/ml.

Fluorescence (at 510 nm for GFP and 460 nm for BFP) was recorded in a Perkin-Elmer 650-40 fluorescence spectrophotometer by using excitation wavelengths of 450 nm and 360 nm for GPF and BFP respectively. Fluorescence was measured in 1 ml samples using dilutions when necessary. Both enzymatic activities and fluorescence were determined in triplicate.

Quantitative protein analysis

Samples of bacterial cultures (10 ml) were low-speed centrifuged (15 min at 12000 g) to harvest the cells. For protein quantification in soluble cell fractions, samples were resuspended in 400 μl of Z buffer without β-mercaptoethanol [23] with one tablet of protease inhibitor cocktail (Roche, ref. 1 836 170) per 10 ml buffer. Such mixtures, once jacketed in ice, were sonicated for 5 min (or longer when required to achieve a complete disruption) at 50 W under 0.5 s cycles as described [21], and centrifuged for 15 min at 12000 g. The supernatant was mixed with denaturing buffer at appropriate ratios [24]. For the determination of inclusion body protein, these structures were purified by repeated detergent washing as described [19] and resuspended in denaturing buffer [24]. After boiling for 20 min, appropriate sample volumes were loaded onto denaturing gels. For Western blot, polyclonal antibodies specific for each protein were used as previously described [17]. Dried blots were scanned at high resolution and bands quantified by using the Quantity One software from Bio Rad, by using appropriate protein dilutions of known concentration as controls. Determinations were always done within the linear range and they were used to calculate the specific activity values.

Conformational analysis by FTIR spectroscopy

For FTIR spectroscopy analysis, purified inclusion bodies were dried for two hours in a Seepd-Vac system before analysis to reduce water interference in the infrared spectra. The FTIR spectrum of the dry samples was analysed directly in a Bruker Tensor FTIR spectrometer. All processing procedures were carried out so as to optimise the quality of the spectrum in the amide I region, between 1600 cm^{-1} and 1700 cm^{-1}. Second derivatives of the amide I band spectra were used to determine the frequencies at which the different spectral components were located. A general description of FTIR procedures can be found elsewhere [9,10].

Abbreviations

BFP blue fluorescent protein

CPRG phenol red β-D-galactopyranoside

FMDV foot-and-mouth disease virus

FTIR fourier transform infrared

GFP green fluorescent protein

HDHFR human dihydropholate reductase

IPTG isopropyl-β-D-thiogalactopyranoside

ONPG ortho-nitrophenyl β-D-galactopyranoside

Authors' contributions

EGF performed most of the experiments and prepared the final data and figures. NGM, A. Vera and AA analysed protein amounts by Western blot, RMF performed enzyme kinetics, MM performed part of optical microscopy analysis and SV part of FTIR analysis and data interpretation. A. Villaverde directed the work and prepared the manuscript.

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References

1. Baneyx F, Mujacic M: **Recombinant protein folding and misfolding in Escherichia coli.** *Nat Biotechnol* 2004, **22:**1399-1408.
2. Strandberg L, Enfors SO: **Factors influencing inclusion body formation in the production of a fused protein in Escherichia coli.** *Appl Environ Microbiol* 1991, **57:**1669-1674.
3. Rinas U, Tsai LB, Lyons D, Fox GM, Stearns G, Fieschko J, Fenton D, Bailey JE: **Cysteine to serine substitutions in basic fibroblast growth factor: effect on inclusion body formation and proteolytic susceptibility during in vitro refolding.** *Biotechnology (N Y)* 1992, **10:**435-440.
4. Baneyx F, Palumbo JL: **Improving heterologous protein folding via molecular chaperone and foldase co-expression.** *Methods Mol Biol* 2003, **205:**171-197.
5. Vallejo LF, Rinas U: **Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins.** *Microb Cell Fact* 2004, **3:**11.
6. Tokatlidis K, Dhurjati P, Millet J, Beguin P, Aubert JP: **High activity of inclusion bodies formed in Escherichia coli overproducing Clostridium thermocellum endoglucanase D.** *FEBS Lett* 1991, **282:**205-208.
7. Garcia-Fruitos E, Carrio MM, Aris A, Villaverde A: **Folding of a misfolding-prone beta-galactosidase in absence of DnaK.** *Biotechnol Bioeng* 2005, **90:**869-875.
8. Worrall DM, Goss NH: **The formation of biologically active beta-galactosidase inclusion bodies in Escherichia coli.** *Aust J Biotechnol* 1989, **3:**28-32.
9. Carrio M, Gonzalez-Montalban N, Vera A, Villaverde A, Ventura S: **Amyloid-like properties of bacterial inclusion bodies.** *J Mol Biol* 2005, **347:**1025-1037.
10. Ami D, Natalello A, Gatti-Lafranconi P, Lotti M, Doglia SM: **Kinetics of inclusion body formation studied in intact cells by FT-IR spectroscopy.** *FEBS Lett* 2005, **579:**3433-3436.
11. Sorensen HP, Mortensen KK: **Soluble expression of recombinant proteins in the cytoplasm of Escherichia coli.** *Microb Cell Fact* 2005, **4:**1.
12. de Marco A, Schroedel A: **Characterization of the aggregates formed during recombinant protein expression in bacteria.** *BMC Biochem* 2005, **6:**10.
13. Chiti F, Taddei N, Baroni F, Capanni C, Stefani M, Ramponi G, Dobson CM: **Kinetic partitioning of protein folding and aggregation.** *Nat Struct Biol* 2002, **9:**137-143.
14. Wright PE, Dyson HJ: **Intrinsically unstructured proteins: reassessing the protein structure-function paradigm.** *J Mol Biol* 1999, **293:**321-331.
15. Waldo GS, Standish BM, Berendzen J, Terwilliger TC: **Rapid protein-folding assay using green fluorescent protein.** *Nat Biotechnol* 1999, **17:**691-695.
16. Carrio MM, Villaverde A: **Protein aggregation as bacterial inclusion bodies is reversible.** *FEBS Lett* 2001, **489:**29-33.
17. Cazorla D, Feliu JX, Villaverde A: **Variable specific activity of Escherichia coli beta-galactosidase in bacterial cells.** *Biotechnol Bioeng* 2001, **72:**255-260.
18. Oberg K, Chrunyk BA, Wetzel R, Fink AL: **Nativelike secondary structure in interleukin-1 beta inclusion bodies by attenuated total reflectance FTIR.** *Biochemistry* 1994, **33:**2628-2634.
19. Carrio MM, Cubarsi R, Villaverde A: **Fine architecture of bacterial inclusion bodies.** *FEBS Lett* 2000, **471:**7-11.
20. Sambrook J, Fritsch E, Maniatis T: *Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY* 1989.
21. Feliu JX, Cubarsi R, Villaverde A: **Optimized release of recombinant proteins by ultrasonication of E. coli cells.** *Biotechnol Bioeng* 1998, **58:**536-540.
22. Ferraz RM, Aris A, Villaverde A: **Profiling the allosteric response of an engineered beta-galactosidase to its effector, anti-HIV antibody.** *Biochem Biophys Res Commun* 2004, **314:**854-860.
23. Miller JH: *Experiments in Molecular Genetics Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY* 1972.
24. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature* 1970, **227:**680-685.
25. Davies JF, Delcamp TJ, Prendergast NJ, Ashford VA, Freisheim JH, Kraut J: **Crystal structures of recombinant human dihydrofolate reductase complexed with folate and 5-deazafolate.** *Biochemistry* 1990, **29:**9467-9479.
26. Sánchez de Groot N, Avilés FX, Vendrell J, Ventura S: **Mutagenesis of the central hydrophobic cluster in Ab42 Alzheimer's peptide. Simple rules to predict the aggregation propensities of polypeptides.** *submitted* 2005.

7. REFERENCES

1998. The importance of simple / rapid assays in HIV testing. WHO/UNAIDS recommendations. Weekly epidemiological report, 73, 321-328.

2005a. HIV diagnostic testing utilization survey report. Association of Public Health Laboratories.

2005b. HIV sequence compendium 2005. Los Alamos database.

2005c. WO/2005/063282. (www. wipo. int/pctdb/en/wo. jsp?WO=2005%2F063282&IA=WO2005%2F063282&DISPLAY=DESC).

Aalberse,R.C., Schuurman,J., 2002. IgG4 breaking the rules. Immunology, 105, 9-19.

Aalberse,R.C., Van Milligen,F., Tan,K.Y., Stapel,S.O., 1993. Allergen-specific IgG4 in atopic disease. Allergy, 48, 559-569.

Abbas,A., Vasilescu,A., Do,H., Hendel,H., Maachi,M., Goutalier,F.X., Regulier,E.G., Rappaport,J., Matsuda,F., Therwath,A., Aucouturier,P., Zagury,J.F., 2005. Analysis of IGG and IGG4 in HIV-1 seropositive patients and correlation with biological and genetic markers. Biomed. Pharmacother., 59, 38-46.

Alcalá,P., Ferrer-Miralles,N., Villaverde,A., 2002. Co-activation of antibody-responsive, enzymatic sensors by a recombinant scFv antibody fragment produced in *E. coli.* Biotechnology Letters, 24, 1543-1551.

Alcala,P., Feliu,J.X., Aris,A., Villaverde,A., 2001. Efficient accommodation of recombinant, foot-and-mouth disease virus RGD peptides to cell-surface integrins. Biochem Biophys Res Commun, 285, 201-206.

Alcami,J., 2004. [Advances in the immunopathology of HIV infection]. Enferm. Infecc. Microbiol. Clin., 22, 486-496.

Aline,F., Brand,D., Bout,D., Pierre,J., Fouquenet,D., Verrier,B., Dimier-Poisson,I., 2007. Generation of specific Th1 and CD8+ T cell responses by immunization with mouse CD8+ dendritic cells loaded with HIV-1 viral lysate or envelope glycoproteins. Microbes. Infect., 9, 536-543.

Allain,J.P., Laurian,Y., Einstein,M.H., Braun,B.P., Delaney,S.R., Stephens,J.E., Daluga,C.K., Dahlen,S.J., Knigge,K.M., 1991. Monitoring of specific antibodies to human immunodeficiency virus structural proteins: clinical significance. Blood, 77, 1118-1123.

Alvaro,G., Fernandez-Lafuente,R., Blanco,R.M., Guisan,J.M., 1990. Immobilization-stabilization of penicillin G acylase from Escherichia coli. Appl. Biochem Biotechnol, 26, 181-195.

Ambrose,Z., KewalRamani,V.N., Bieniasz,P.D., Hatziioannou,T., 2007. HIV/AIDS: in search of an animal model. Trends Biotechnol., 25, 333-337.

Aris,A., Villaverde,A., 2004. Modular protein engineering for non-viral gene therapy. Trends Biotechnol, 22, 371-377.

Ausubel,F., Kingston,R., Moore,D., Seidman,J., Smith,J., Struhl,K., 1987. Current protocols in molecular biology. John Wiley and Sons Inc.,New York, NY.

Ball,J.C., Puckett,L.G., Bachas,L.G., 2003. Covalent immobilization of beta-galactosidase onto a gold-coated magnetoelastic transducer via a self-assembled monolayer: toward a magnetoelastic biosensor. Anal. Chem., 75, 6932-6937.

Baum,E.Z., Bebernitz,G.A., Gluzman,Y., 1990. beta-Galactosidase containing a human immunodeficiency virus protease cleavage site is cleaved and inactivated by human immunodeficiency virus protease. Proc. Natl. Acad. Sci. U. S. A, 87, 10023-10027.

Bckstrom,M., Holmgren,J., Schodel,F., Lebens,M., 1995. Characterization of an internal permissive site in the cholera toxin B-subunit and insertion of epitopes from human immunodeficiency virus-1, hepatitis B virus and enterotoxigenic Escherichia coli. Gene, 165, 163-171.

Becker,Y., 2004. HIV-1 induced AIDS is an allergy and the allergen is the Shed gp120--a review, hypothesis, and implications. Virus Genes, 28, 319-331.

Benito,A., Feliu,J.X., Villaverde,A., 1996. Beta-galactosidase enzymatic activity as a molecular probe to detect specific antibodies. J Biol Chem, 271, 21251-21256.

Benito,A., Mateu,M.G., Villaverde,A., 1995. Improved mimicry of a foot-and-mouth disease virus antigenic site by a viral peptide displayed on beta-galactosidase surface. Biotechnology (N Y), 13, 801-804.

Benito,A., Valero,F., Lafuente,J., Vidal,M., Cairo,J., Sola,C., Villaverde,A., 1993. Uses of beta-galactosidase tag in on-line monitoring production of fusion proteins and gene expression in Escherichia coli. Enzyme Microb Technol, 15, 66-71.

Benito,A., Vidal,M., Villaverde,A., 1993. Enhanced production of pL-controlled recombinant proteins and plasmid stability in Escherichia coli RecA+ strains. J. Biotechnol., 29, 299-306.

Benito,A., Villaverde,A., 1994. Insertion of a 27 amino acid viral peptide in different zones of Escherichia coli beta-galactosidase: effects on the enzyme activity. FEMS Microbiol Lett, 123, 107-112.

Benito,J.M., Lopez,M., Soriano,V., 2004. The role of CD8+ T-cell response in HIV infection. AIDS Rev., 6, 79-88.

Berggard,T., Linse,S., James,P., 2007. Methods for the detection and analysis of protein-protein interactions. Proteomics, 7, 2833-2842.

Betancor L, Luckarift HR, Seo JH, Brand O, Spain JC, 2008. Three-dimensional immobilization of beta-galactosidase on a silicon surface. Biotechnol Bioeng, 99, 261-267.

Binley,J.M., Trkola,A., Ketas,T., Schiller,D., Clas,B., Little,S., Richman,D., Hurley,A., Markowitz,M., Moore,J.P., 2000. The effect of highly active antiretroviral therapy on binding and neutralizing antibody responses to human immunodeficiency virus type 1 infection. J. Infect. Dis., 182, 945-949.

Blackman,M.J., Corrie,J.E., Croney,J.C., Kelly,G., Eccleston,J.F., Jameson,D.M., 2002. Structural and biochemical characterization of a fluorogenic rhodamine-labeled malarial protease substrate. Biochemistry, 41, 12244-12252.

Blanco,R.M., Bastida,A., Cuesta,C., Alvaro,G., Fernandez-Lafuente,R., Rosell,C.M., Guisan,J.M., 1991. Immobilization-stabilization of proteases

as a tool to improve the industrial design of peptide synthesis. Biomed. Biochim. Acta, 50, S110-S113.

Blanco,R., Guisan,J.M., 1989. Stabilization of enzymes by multipoint covalent attachment to agarose-aldehyde gels. Borohydride reduction of tripsin-agarose derivatives. Enzyme and Microbial Technology, 11, 360-366.

Brainina,K., Kozitsina,A., Beikin,J., 2003. Electrochemical immunosensor for Forest-Spring encephalitis based on protein A labeled with colloidal gold. Anal. Bioanal. Chem., 376, 481-485.

Branson,B., 2007. State of the art for diagnosis of HIV infection. CID, 45 (suppl. 4), S221-S225.

Breaker,R.R., 2002. Engineered allosteric ribozymes as biosensor components. Curr Opin Biotechnol, 13, 31-39.

Brennan,C., Christianson,K., Surowy,T., Mandecki,W., 1994. Modulation of enzyme activity by antibody binding to an alkaline phosphatase-epitope hybrid protein. Protein Eng, 7, 509-514.

Brennan,C.A., Christianson,K., La Fleur,M.A., Mandecki,W., 1995. A molecular sensor system based on genetically engineered alkaline phosphatase. Proc Natl Acad Sci U S A, 92, 5783-5787.

Broliden,P.A., von Gegerfelt,A., Clapham,P., Rosen,J., Fenyo,E.M., Wahren,B., Broliden,K., 1992. Identification of human neutralization-inducing regions of the human immunodeficiency virus type 1 envelope glycoproteins. Proc. Natl. Acad. Sci. U. S. A, 89, 461-465.

Burkly,L.C., Olson,D., Shapiro,R., Winkler,G., Rosa,J.J., Thomas,D.W., Williams,C., Chisholm,P., 1992. Inhibition of HIV infection by a novel CD4 domain 2-specific monoclonal antibody. Dissecting the basis for its inhibitory effect on HIV-induced cell fusion. J. Immunol., 149, 1779-1787.

Burton,G.F., Keele,B.F., Estes,J.D., Thacker,T.C., Gartner,S., 2002. Follicular dendritic cell contributions to HIV pathogenesis. Semin. Immunol., 14, 275-284.

Cabana,M., Fernandez,G., Parera,M., Clotet,B., Martinez,M.A., 2002. Catalytic efficiency and phenotype of HIV-1 proteases encoding single critical resistance substitutions. Virology, 300, 71-78.

Cao,Y., Qin,L., Zhang,L., Safrit,J., Ho,D.D., 1995. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. N. Engl. J. Med., 332, 201-208.

Casadaban,M.J., Chou,J., Cohen,S.N., 1980. In vitro gene fusions that join an enzymatically active beta-galactosidase segment to amino-terminal fragments of exogenous proteins: Escherichia coli plasmid vectors for the detection and cloning of translational initiation signals. J Bacteriol, 143, 971-980.

Casadaban,M.J., Martinez-Arias,A., Shapira,S.K., Chou,J., 1983. Beta-galactosidase gene fusions for analyzing gene expression in escherichia coli and yeast. Methods Enzymol, 100, 293-308.

Cavacini,L.A., Emes,C.L., Power,J., Desharnais,F.D., Duval,M., Montefiori,D., Posner,M.R., 1995. Influence of heavy chain constant regions on antigen binding and HIV-1 neutralization by a human monoclonal antibody. J. Immunol., 155, 3638-3644.

Cavacini,L.A., Kuhrt,D., Duval,M., Mayer,K., Posner,M.R., 2003. Binding and neutralization activity of human IgG1 and IgG3 from serum of HIV-infected individuals. AIDS Res. Hum. Retroviruses, 19, 785-792.

Cazorla,D., Feliu,J.X., Ferrer-Miralles,N., Villaverde,A., 2002. Tailoring molecular sensing for peptide displaying engineered enzymes. Biotechnology Letters, **24**, 467-477.

Chao,Y.P., Chern,J.T., Wen,C.S., Fu,H., 2002. Construction and characterization of thermo-inducible vectors derived from heat-sensitive lacI genes in combination with the T7 A1 promoter. Biotechnol Bioeng., 79, 1-8.

Charbit,A., Ronco,J., Michel,V., Werts,C., Hofnung,M., 1991. Permissive sites and topology of an outer membrane protein with a reporter epitope. J. Bacteriol., 173, 262-275.

Chargelegue,D., Stanley,C.M., O'Toole,C.M., Colvin,B.T., Steward,M.W., 1995. The affinity of IgG antibodies to gag p24 and p17 in HIV-1-infected patients correlates with disease progression. Clin Exp. Immunol, 99, 175-181.

Chin,C.D., Linder,V., Sia,S.K., 2007. Lab-on-a-chip devices for global health: past studies and future opportunities. Lab Chip., 7, 41-57.

Chudakov,D.M., Lukyanov,S., Lukyanov,K.A., 2005. Fluorescent proteins as a toolkit for in vivo imaging. Trends Biotechnol., 23, 605-613.

Coeffier,E., Clement,J.M., Cussac,V., Khodaei-Boorane,N., Jehanno,M., Rojas,M., Dridi,A., Latour,M., El Habib,R., Barre-Sinoussi,F., Hofnung,M., Leclerc,C., 2000. Antigenicity and immunogenicity of the HIV-1 gp41 epitope ELDKWA inserted into permissive sites of the MalE protein. Vaccine, 19, 684-693.

Coffin,J.M., Hughes,S.H., Varmus,H.E., 1997. Retroviruses at the National Center for Biotechnology Information. Cold Spring Harbor Laboratory Press.

Collings,A., Caruso,F., 1997. Biosensors: Recent advances. Rep. Prog. Phys., 60, 1397-1445.

Cooper,M.A., 2003. Biosensor profiling of molecular interactions in pharmacology. Curr. Opin. Pharmacol., 3, 557-562.

de Martino,M., Rossi,M.E., Azzari,C., Chiarelli,F., Galli,L., Vierucci,A., 1999. Low IgG3 and high IgG4 subclass levels in children with advanced human immunodeficiency virus-type 1 infection and elevated IgE levels. Ann. Allergy Asthma Immunol., 83, 160-164.

de Rosny,E., Vassell,R., Jiang,S., Kunert,R., Weiss,C.D., 2004. Binding of the 2F5 monoclonal antibody to native and fusion-intermediate forms of human immunodeficiency virus type 1 gp41: implications for fusion-inducing conformational changes. J. Virol., 78, 2627-2631.

Doi,N., Yanagawa,H., 1999a. Design of generic biosensors based on green fluorescent proteins with allosteric sites by directed evolution. FEBS Lett, 453, 305-307.

Doi,N., Yanagawa,H., 1999b. Insertional gene fusion technology. FEBS Lett, 457, 1-4.

Doi,N., Yanagawa,H., 2002. Evolutionary design of generic green fluorescent

protein biosensors. Methods Mol Biol, 183, 49-55.

Dube,D.K., Sherman,M.P., Saksena,N.K., Bryz-Gornia,V., Mendelson,J., Love,J., Arnold,C.B., Spicer,T., Dube,S., Glaser,J.B., ., 1993. Genetic heterogeneity in human T-cell leukemia/lymphoma virus type II. J. Virol., 67, 1175-1184.

Ehrmann,M., Clausen,T., 2004. Proteolysis as a regulatory mechanism. Annu. Rev. Genet., 38, 709-724.

Feliu,J.X., Ferrer-Miralles,N., Villaverde,A., 2000. Beta-Galactosidase-based enzymatic biosensors. Recent Res. Devel. Biotech. & Bioeng., 3, 21-31.

Feliu,J.X., Ferrer-Miralles,N., Blanco,E., Cazorla,D., Sobrino,F., Villaverde,A., 2002. Enhanced response to antibody binding in engineered beta-galactosidase enzymatic sensors. Biochim Biophys Acta, 1596, 212-224.

Feliu,J.X., Ramirez,E., Villaverde,A., 1998. Distinct mechanisms of antibody-mediated enzymatic reactivation in beta-galactosidase molecular sensors. FEBS Lett, 438, 267-271.

Feliu,J.X., Villaverde,A., 1998. Engineering of solvent-exposed loops in Escherichia coli beta-galactosidase. FEBS Lett, 434, 23-27.

Ferraz,R.M., Vera,A., Aris,A., Villaverde,A., 2006. Insertional protein engineering for analytical molecular sensing. Microb. Cell Fact., 5, 15.

Ferrer-Miralles,N., Feliu,J.X., Vandevuer,S., Muller,A., Cabrera-Crespo,J., Ortmans,I., Hoffmann,F., Cazorla,D., Rinas,U., Prevost,M., Villaverde,A., 2001. Engineering regulable Escherichia coli beta-galactosidases as biosensors for anti-HIV antibody detection in human sera. J Biol Chem, 276, 40087-40095.

Ferrer-Miralles,N., Feliu,J.X., Villaverde,A., 2000. Molecular mechanisms for antibody-mediated modulation of peptide-displaying enzyme sensors. Biochem Biophys Res Commun, 275, 360-364.

Fiorentini,S., Marini,E., Bozzo,L., Trainini,L., Saadoune,L., Avolio,M., Pontillo,A., Bonfanti,C., Sarmientos,P., Caruso,A., 2004. Preclinical studies on immunogenicity of the HIV-1 p17-based synthetic peptide AT20-KLH. Biopolymers, 76, 334-343.

Fletcher,M., Miguez-Burbano,M.J., Shor-Posner,G., Lopez,V., Lai,H., Baum,M.K., 2000. Diagnosis of human immunodeficiency virus infection using an immunoglobulin E-based assay. Clin. Diagn. Lab Immunol., 7, 55-57.

Gabig-Ciminska,M., Holmgren,A., Andresen,H., Bundvig,B.K., Wumpelmann,M., Albers,J., Hintsche,R., Breitenstein,A., Neubauer,P., Los,M., Czyz,A., Wegrzyn,G., Silfversparre,G., Jurgen,B., Schweder,T., Enfors,S.O., 2004. Electric chips for rapid detection and quantification of nucleic acids. Biosens. Bioelectron., 19, 537-546.

Gauglitz,G., Proll,G., 2008. Strategies for label-free optical detection. Adv. Biochem. Eng Biotechnol., 109, 395-432.

Geddie,M.L., O'Loughlin,T.L., Woods,K.K., Matsumura,I., 2005. Rational design of p53, an intrinsically unstructured protein, for the fabrication of novel molecular sensors. J. Biol. Chem., 280, 35641-35646.

Geldmacher,C., Currier,J.R., Herrmann,E., Haule,A., Kuta,E., McCutchan,F., Njovu,L., Geis,S., Hoffmann,O., Maboko,L.,

Williamson,C., Birx,D., Meyerhans,A., Cox,J., Hoelscher,M., 2007. CD8 T-cell recognition of multiple epitopes within specific Gag regions is associated with maintenance of a low steady-state viremia in human immunodeficiency virus type 1-seropositive patients. J. Virol., 81, 2440-2448.

Goh,Y.Y., Frecer,V., Ho,B., Ding,J.L., 2002. Rational design of green fluorescent protein mutants as biosensor for bacterial endotoxin. Protein Eng, 15, 493-502.

Gomara,M.J., Ercilla,G., Alsina,M.A., Haro,I., 2000. Assessment of synthetic peptides for hepatitis A diagnosis using biosensor technology. J. Immunol. Methods, 246, 13-24.

Gong,J.-L., Gong,F.-C., Zeng,G.-M., Shen,G.-L., Yu,R.-Q., 2007. An amperometric immunosensor for the Newcastle disease antibody assay. Analytical Letters, 36 (2), 287-302.

Goulet,B., Nepveu,A., 2004. Complete and limited proteolysis in cell cycle progression. Cell Cycle, 3, 986-989.

Greenwald,J.L., 2006. Routine rapid HIV testing in hospitals. Journal of Hospital Medicine, 1, 106-112.

Guilbault,G.G., Pravda,M., Kreuzer,M., O'Sullivan,C.K., 2007. Biosensors: 42 years and counting. Analytical Letters, 37, 1481-1496.

Guisán,J., 1988. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. Enzyme and Microbial Technology, 10, 375-382.

Guntas,G., Ostermeier,M., 2004. Creation of an allosteric enzyme by domain insertion. J. Mol. Biol., 336, 263-273.

Haasnoot,W., Marchesini,G.R., Koopal,K., 2006. Spreeta-based biosensor immunoassays to detect fraudulent adulteration in milk and milk powder. J. AOAC Int., 89, 849-855.

Habauzit,D., Chopineau,J., Roig,B., 2007. SPR based biosensors: a tool for biodetection of hormonal compounds. Anal. Bioanal. Chem., 387, 1215-1223.

Hamano,H., Kawa,S., Horiuchi,A., Unno,H., Furuya,N., Akamatsu,T., Fukushima,M., Nikaido,T., Nakayama,K., Usuda,N., Kiyosawa,K., 2001. High serum IgG4 concentrations in patients with sclerosing pancreatitis. N. Engl. J. Med., 344, 732-738.

Hamman,B.D., Oleinikov,A.V., Jokhadze,G.G., Bochkariov,D.E., Traut,R.R., Jameson,D.M., 1996. Tetramethylrhodamine dimer formation as a spectroscopic probe of the conformation of Escherichia coli ribosomal protein L7/L12 dimers. J. Biol. Chem., 271, 7568-7573.

Hashida,S., Hashinaka,K., Ishikawa,S., Ishikawa,E., 1997. More reliable diagnosis of infection with human immunodeficiency virus type 1 (HIV-1) by detection of antibody IgGs to pol and gag proteins of HIV-1 and p24 antigen of HIV-1 in urine, saliva, and/or serum with highly sensitive and specific enzyme immunoassay (immune complex transfer enzyme immunoassay): a review. J. Clin. Lab Anal., 11, 267-286.

Hashida,S., Hashinaka,K., Nishikata,I., Oka,S., Shimada,K., Saito,A., Takamizawa,A., Shinagawa,H., Ishikawa,E., 1996. Shortening of the window period in diagnosis of HIV-1 infection by simultaneous detection of p24 antigen and antibody IgG to p17 and reverse transcriptase in serum with ultrasensitive

enzyme immunoassay. J. Virol. Methods, 62, 43-53.

Hay,C., Rosenberg,E., 1998. Immunologic response to HIV. AIDS Clin. Care, 10, 1-3.

Heeney,J.L., Plotkin,S.A., 2006. Immunological correlates of protection from HIV infection and disease. Nat. Immunol., 7, 1281-1284.

Hilt,W., 2004. Targets of programmed destruction: a primer to regulatory proteolysis in yeast. Cell Mol. Life Sci., 61, 1615-1632.

Hiraga,K., Yamagishi,A., Oshima,T., 2004. Mapping of unit boundaries of a protein: exhaustive search for permissive sites for duplication by complementation analysis of random fragment libraries of tryptophan synthase alpha subunit. J. Mol. Biol., 335, 1093-1104.

Hu,Q., Mahmood,N., Shattock,R.J., 2007. High-mannose-specific deglycosylation of HIV-1 gp120 induced by resistance to cyanovirin-N and the impact on antibody neutralization. Virology, 368, 145-154.

Huber,R.E., Kurz,G., Wallenfels,K., 1976. A quantitation of the factors which affect the hydrolase and transgalactosylase activities of beta-galactosidase (E. coli) on lactose. Biochemistry, 15, 1994-2001.

Huidobro-Toro,J.P., Lorca,R.A., Coddou,C., 2008. Trace metals in the brain: allosteric modulators of ligand-gated receptor channels, the case of ATP-gated P2X receptors. Eur. Biophys. J., 37, 301-314.

id-Peralta,L., Grangeot-Keros,L., Rudent,A., Ngo-Giang-Huong,N., Krzysiek,R., Goujard,C., Deveau,C., Le,G.M., Meyer,L., Emilie,D., Rouzioux,C., 2006. Impact of highly active antiretroviral therapy on the maturation of anti-HIV-1 antibodies during primary HIV-1 infection. HIV. Med., 7, 514-519.

Isa,M.B., Martinez,L., Giordano,M., Passeggi,C., de Wolff,M.C., Nates,S., 2002. Comparison of immunoglobulin G subclass profiles induced by measles virus in vaccinated and naturally infected individuals. Clin. Diagn. Lab Immunol., 9, 693-697.

Isa,M.B., Martinez,L.C., Ferreyra,L.J., Giordano,M.O., Barril,P.A., Massachessi,G., Nates,S.V., 2006. Measles virus-specific IgG4 antibody titer as a serologic marker of post-vaccinal immune response. Viral Immunol., 19, 335-339.

Jacob,F., Monod,J., 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol Biol., 3, 318-356.

Jacobson,R.H., Matthews,B.W., 1992. Crystallization of beta-galactosidase from Escherichia coli. J Mol Biol, 223, 1177-1182.

Jacobson,R.H., Zhang,X.J., DuBose,R.F., Matthews,B.W., 1994. Three-dimensional structure of beta-galactosidase from E. coli. Nature, 369, 761-766.

Janvier,B., Baillou,A., Archinard,P., Mounier,M., Mandrand,B., Goudeau,A., Barin,F., 1991. Immune response to a major epitope of p24 during infection with human immunodeficiency virus type 1 and implications for diagnosis and prognosis. J. Clin. Microbiol., 29, 488-492.

Joag,S.V., Stephens,E.B., Narayan,O., 1996. Lentiviruses. 1977-1996.

Joller-Jemelka,H.I., Joller,P.W., Muller,F., Schupbach,J., Grob,P.J., 1987. Anti-HIV IgM antibody analysis during early

manifestations of HIV infections. AIDS, 1, 45-47.

Ju,H.X., Ye,Y.K., Zhao,J.H., Zhu,Y.L., 2003. Hybridization biosensor using di(2,2'-bipyridine)osmium (III) as electrochemical indicator for detection of polymerase chain reaction product of hepatitis B virus DNA. Anal. Biochem., 313, 255-261.

Khalife,J., Guy,B., Capron,M., Kieny,M.P., Ameisen,J.C., Montagnier,L., Lecocq,J.P., Capron,A., 1988. Isotypic restriction of the antibody response to human immunodeficiency virus. AIDS Res. Hum. Retroviruses, 4, 3-9.

Kinloch-De Loes,S., Perrin,L., 1995. Therapeutic interventions in primary HIV infection. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol., 10 Suppl 1, S69-S76.

Klasse,P.J., 1996. Physico-chemical analyses of humoral immune response to HIV-1: Quantification od antibodies, their binding to viral antigens and neutralization of viral infectivity. In: Korber B, Brander C, Haynes B, Moore JP, Walker BD, D'Sousa PM, Myers G (Eds.), Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM., IV-22-IV-49.

Klasse,P.J., Blomberg,J., Pipkorn,R., 1990. Differential IgG subclass responses to epitopes in transmembrane protein of HIV-1. Viral Immunol., 3, 89-98.

Klenerman,P., Wu,Y., Phillips,R., 2002. HIV: current opinion in escapology. Curr. Opin. Microbiol., 5, 408-413.

Koch,M., Pancera,M., Kwong,P.D., Kolchinsky,P., Grundner,C., Wang,L., Hendrickson,W.A., Sodroski,J., Wyatt,R., 2003. Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition. Virology, 313, 387-400.

Kohl,T., Heinze,K.G., Kuhlemann,R., Koltermann,A., Schwille,P., 2002. A protease assay for two-photon crosscorrelation and FRET analysis based solely on fluorescent proteins. Proc. Natl. Acad. Sci. U. S. A, 99, 12161-12166.

Kohn,J.E., Plaxco,K.W., 2005. Engineering a signal transduction mechanism for protein-based biosensors. Proc. Natl. Acad. Sci. U. S. A, 102, 10841-10845.

Kozinetz,C.A., Matusa,R., Ruta,S., Hacker,C.S., Cernescu,C., Cazacu,A., 2005. Alternatives to HIV-RNA and CD4 count to monitor HIV disease progression: a prospective cohort study in Romania. J. Med. Virol., 77, 159-163.

Kwakye,S., Baeumner,A., 2003. A microfluidic biosensor based on nucleic acid sequence recognition. Anal. Bioanal. Chem., 376, 1062-1068.

Kwong,P.D., Doyle,M.L., Casper,D.J., Cicala,C., Leavitt,S.A., Majeed,S., Steenbeke,T.D., Venturi,M., Chaiken,I., Fung,M., Katinger,H., Parren,P.W., Robinson,J., Van Ryk,D., Wang,L., Burton,D.R., Freire,E., Wyatt,R., Sodroski,J., Hendrickson,W.A., Arthos,J., 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature, 420, 678-682.

Lee,W.E., Thompson,H.G., Hall,J.G., Fulton,R.E., Wong,J.P., 1993. Rapid immunofiltration assay of Newcastle disease virus using a silicon sensor. J. Immunol. Methods, 166, 123-131.

Legendre,D., Soumillion,P., Fastrez,J., 1999. Engineering a regulatable enzyme for homogeneous immunoassays. Nat Biotechnol, 17, 67-72.

Legendre,D., Vucic,B., Hougardy,V., Girboux,A.L., Henrioul,C., Van Haute,J., Soumillion,P., Fastrez,J., 2002. TEM-1 beta-lactamase as a scaffold for protein recognition and assay. Protein Sci, 11, 1506-1518.

Leonard,P., Hearty,S., Dunne,L., Quinn,J., Chakraborty,T., O'Kennedy,R., 2007. Advances in biosensors for detection of pathogens in food and water. Enzyme and Microbial Technology, 32, 3-13.

Lichterfeld,M., Yu,X.G., Le Gall,S., Altfeld,M., 2005. Immunodominance of HIV-1-specific CD8(+) T-cell responses in acute HIV-1 infection: at the crossroads of viral and host genetics. Trends Immunol., 26, 166-171.

Ljunggren,K., Broliden,P.A., Morfeldt-Manson,L., Jondal,M., Wahren,B., 1988. IgG subclass response to HIV in relation to antibody-dependent cellular cytotoxicity at different clinical stages. Clin. Exp. Immunol., 73, 343-347.

Los,M., Los,J.M., Blohm,L., Spillner,E., Grunwald,T., Albers,J., Hintsche,R., Wegrzyn,G., 2005. Rapid detection of viruses using electrical biochips and anti-virion sera. Lett. Appl. Microbiol., 40, 479-485.

Lottersberger,J., Salvetti,J.L., Tonarelli,G., 2003. [Evaluation of synthetic peptides for the detection of antibodies against human immunodeficiency virus]. Rev. Argent Microbiol., 35, 149-155.

Maeda,Y., Yusa,K., Harada,S., 2007. Altered sensitivity of an R5X4 HIV-1 strain 89.6 to coreceptor inhibitors by a single amino acid substitution in the V3 region of gp120. Antiviral Res..

Maini,M.K., Casorati,G., Dellabona,P., Wack,A., Beverley,P.C., 1999. T-cell clonality in immune responses. Immunol. Today, 20, 262-266.

Manoil,C., Bailey,J., 1997. A simple screen for permissive sites in proteins: analysis of Escherichia coli lac permease. J. Mol. Biol., 267, 250-263.

Margolick,J.B., Munoz,A., Donnenberg,A.D., Park,L.P., Galai,N., Giorgi,J.V., O'Gorman,M.R., Ferbas,J., 1995. Failure of T-cell homeostasis preceding AIDS in HIV-1 infection. The Multicenter AIDS Cohort Study. Nat. Med., 1, 674-680.

Martineau,P., Guillet,J.G., Leclerc,C., Hofnung,M., 1992. Expression of heterologous peptides at two permissive sites of the MalE protein: antigenicity and immunogenicity of foreign B-cell and T-cell epitopes. Gene, 113, 35-46.

Martinez,M.A., Cabana,M., Parera,M., Gutierrez,A., Este,J.A., Clotet,B., 2000. A bacteriophage lambda-based genetic screen for characterization of the activity and phenotype of the human immunodeficiency virus type 1 protease. Antimicrob. Agents Chemother., 44, 1132-1139.

Martinez,M.A., Clotet,B., 2003. Genetic screen for monitoring hepatitis C virus NS3 serine protease activity. Antimicrob. Agents Chemother., 47, 1760-1765.

Martinez-Bilbao,M., Huber,R.E., 1996. The activation of beta-galactosidase (E. coli) by Mg(2+) at lower pH values. Biochem Cell Biol, 74, 295-298.

Marvin,J.S., Hellinga,H.W., 2001. Conversion of a maltose receptor into a zinc biosensor by computational design. Proc. Natl. Acad. Sci. U. S. A, 98, 4955-4960.

Mathonet,P., Barrios,H., Soumillion,P., Fastrez,J., 2006. Selection of allosteric beta-lactamase mutants featuring an activity regulation by transition metal ions. Protein Sci., 15, 2335-2343.

Matthews,B.W., 2005. The Structure of E.coli b-galactosidase. C. R. Biologies, 328, 549-556.

McCaffrey,R.A., Saunders,C., Hensel,M., Stamatatos,L., 2004. N-linked glycosylation of the V3 loop and the immunologically silent face of gp120 protects human immunodeficiency virus type 1 SF162 from neutralization by anti-gp120 and anti-gp41 antibodies. J. Virol., 78, 3279-3295.

Meric,B., Kerman,K., Ozkan,D., Kara,P., Erensoy,S., Akarca,U.S., Mascini,M., Ozsoz,M., 2007. Electrochemical DNA biosensor for the detection of TT and Hepatitis B virus from PCR amplified real samples by using methylene blue. Talanta, 56, 837-846.

Miguez-Burbano,M.J., Shor-Posner,G., Fletcher,M.A., Lu,Y., Moreno,J.N., Carcamo,C., Page,B., Quesada,J., Sauberlich,H., Baum,M.K., 1995. Immunoglobulin E levels in relationship to HIV-1 disease, route of infection, and vitamin E status. Allergy, 50, 157-161.

Miller,J.H., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY..

Miranda,L.R., Duval,M., Doherty,H., Seaman,M.S., Posner,M.R., Cavacini,L.A., 2007. The neutralization properties of a HIV-specific antibody are markedly altered by glycosylation events outside the antigen-binding domain. J. Immunol., 178, 7132-7138.

Morrow,W.J., Williams,W.M., Whalley,A.S., Ryskamp,T., Newman,R., Kang,C.Y., Chamat,S., Kohler,H., Kieber-Emmons,T., 1992. Synthetic peptides from a conserved region of gp120 induce broadly reactive anti-HIV responses. Immunology, 75, 557-564.

Muginova,S.V., Zhavoronkova,A.M., Polyakov,A.E., Shekhovtsova,T.N., 2007. Application of alkaline phosphatases from different sources in pharmaceutical and clinical analysis for the determination of their cofactors; zinc and magnesium ions. Anal. Sci., 23, 357-363.

Muller,F., Muller,K.H., 1988. Detection of anti-HIV-1 immunoglobulin M antibodies in patients with serologically proved HIV-1 infection. Infection, 16, 115-118.

Myszka,D.G., Sweet,R.W., Hensley,P., Brigham-Burke,M., Kwong,P.D., Hendrickson,W.A., Wyatt,R., Sodroski,J., Doyle,M.L., 2000. Energetics of the HIV gp120-CD4 binding reaction. Proc. Natl. Acad. Sci. U. S. A, 97, 9026-9031.

Ngo-Giang-Huong,N., Candotti,D., Goubar,A., Autran,B., Maynart,M., Sicard,D., Clauvel,J.P., Agut,H., Costagliola,D., Rouzioux,C., 2001. HIV type 1-specific IgG2 antibodies: markers of helper T cell type 1 response and prognostic marker of long-term nonprogression. AIDS Res Hum Retroviruses, 17, 1435-1446.

Ostermeier,M., 2005. Engineering allosteric protein switches by domain insertion. Protein Eng Des Sel, 18, 359-364.

Parera,M., Clotet,B., Martinez,M.A., 2004b. Genetic screen for monitoring severe acute respiratory syndrome coronavirus 3C-like protease. J. Virol., 78, 14057-14061.

Parera,M., Clotet,B., Martinez,M.A., 2004a. Genetic screen for monitoring severe acute respiratory syndrome coronavirus 3C-like protease. J. Virol., 78, 14057-14061.

Park,E.J., Gorny,M.K., Zolla-Pazner,S., Quinnan,G.V., Jr., 2000. A global neutralization resistance phenotype of human immunodeficiency virus type 1 is determined by distinct mechanisms mediating enhanced infectivity and conformational change of the envelope complex. J. Virol., 74, 4183-4191.

Park,E.J., Vujcic,L.K., Anand,R., Theodore,T.S., Quinnan,G.V., Jr., 1998. Mutations in both gp120 and gp41 are responsible for the broad neutralization resistance of variant human immunodeficiency virus type 1 MN to antibodies directed at V3 and non-V3 epitopes. J. Virol., 72, 7099-7107.

Pejcic,B., De Marco,R., Parkinson,G., 2006. The role of biosensors in the detection of emerging infectious diseases. Analyst, 131, 1079-1090.

Pelisek J., Armeanu S, Nikol S., 2000. Evaluation of beta-galactosidase activity in tissue in the presence of blood. J Vasc Res, 37, 585-593.

Pellegrino,M.G., Bluth,M.H., Smith-Norowitz,T., Fikrig,S., Volsky,D.J., Moallem,H., Auci,D.L., Nowakowski,M., Durkin,H.G., 2002. HIV type 1-specific IgE in serum of long-term surviving children inhibits HIV type 1 production in vitro. AIDS Res. Hum. Retroviruses, 18, 363-372.

Pufall,M.A., Graves,B.J., 2002. Autoinhibitory domains: modular effectors of cellular regulation. Annu. Rev. Cell Dev. Biol., 18, 421-462.

Purvis,D., Leonardova,O., Farmakovsky,D., Cherkasov,V., 2003. An ultrasensitive and stable potentiometric immunosensor. Biosens. Bioelectron., 18, 1385-1390.

Ramanathan,K., Danielsson,B., 2001. Principles and applications of thermal biosensors. Biosens. Bioelectron., 16, 417-423.

Ramanathan,K., Rank,M., Svitel,J., Dzgoev,A., Danielsson,B., 1999. The development and applications of thermal biosensors for bioprocess monitoring. Trends Biotechnol, 17, 499-505.

Renard,M., Belkadi,L., Bedouelle,H., 2003. Deriving topological constraints from functional data for the design of reagentless fluorescent immunosensors. J. Mol Biol., 326, 167-175.

Richardson,S.D., 2007. Water analysis: emerging contaminants and current issues. Anal. Chem., 79, 4295-4323.

Rosen,O., Chill,J., Sharon,M., Kessler,N., Mester,B., Zolla-Pazner,S., Anglister,J., 2005. Induced fit in HIV-neutralizing antibody complexes: evidence for alternative conformations of the gp120 V3 loop and the molecular basis for broad neutralization. Biochemistry, 44, 7250-7258.

Rowe-Taitt,C.A., Golden,J.P., Feldstein,M.J., Cras,J.J., Hoffman,K.E., Ligler,F.S., 2000. Array biosensor for detection of biohazards. Biosens. Bioelectron., 14, 785-794.

Sambrook,J., Fritsch,E., Maniatis,T., 1989. Molecular Cloning, A Laboratory Manual, . Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sarkar,P., Pal,P.S., Ghosh,D., Setford,S.J., Tothill,I.E., 2002. Amperometric biosensors for detection of the prostate cancer marker (PSA). Int. J. Pharm., 238, 1-9.

Schauder,B., Blocker,H., Frank,R., McCarthy,J.E., 1987. Inducible expression vectors incorporating the Escherichia coli atpE translational initiation region. Gene, 52, 279-283.

Scheller,F.W., Wollenberger,U., Warsinke,A., Lisdat,F., 2001. Research and development in biosensors. Curr. Opin. Biotechnol, 12, 35-40.

Sester,M., Sester,U., Kohler,H., Schneider,T., Deml,L., Wagner,R., Mueller-Lantzsch,N., Pees,H.W., Meyerhans,A., 2000. Rapid whole blood analysis of virus-specific CD4 and CD8 T cell responses in persistent HIV infection. AIDS, 14, 2653-2660.

Sheppard,N.C., Bates,A.C., Sattentau,Q.J., 2007. A functional human IgM response to HIV-1 Env after immunization with NYVAC HIV C. AIDS, 21, 524-527.

Sheppard,N.C., Davies,S.L., Jeffs,S.A., Vieira,S.M., Sattentau,Q.J., 2007. Production and characterization of high-affinity human monoclonal antibodies to human immunodeficiency virus type 1 envelope glycoproteins in a mouse model expressing human immunoglobulins. Clin. Vaccine Immunol., 14, 157-167.

Simon,V., Ho,D.D., Abdool,K.Q., 2006. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. Lancet, 368, 489-504.

Singh,S., 2007. Sensors--an effective approach for the detection of explosives. J. Hazard. Mater., 144, 15-28.

Tai,D.F., Lin,C.Y., Wu,T.Z., Chen,L.K., 2005. Recognition of dengue virus protein using epitope-mediated molecularly imprinted film. Anal. Chem., 77, 5140-5143.

Tang,D.P., Yuan,R., Chai,Y.Q., Zhong,X., Liu,Y., Dai,J.Y., Zhang,L.Y., 2004. Novel potentiometric immunosensor for hepatitis B surface antigen using a gold nanoparticle-based biomolecular immobilization method. Anal. Biochem, 333, 345-350.

Thevenot,D.R., Toth,K., Durst,R.A., Wilson,G.S., 2001. Electrochemical biosensors: recommended definitions and classification. Biosens. Bioelectron., 16, 121-131.

Tucker,C.L., Fields,S., 2001. A yeast sensor of ligand binding. Nat. Biotechnol., 19, 1042-1046.

Ullmann,A., 2001. Escherichia coli Lactose Operon. Encyclopedia of life sciences.

Ullmann,A., 1984. One-step purification of hybrid proteins which have beta-galactosidase activity. Gene, 29, 27-31.

Uludag,Y., Li,X., Coleman,H., Efstathiou,S., Cooper,M.A., 2008. Direct acoustic profiling of DNA hybridisation using HSV type 1 viral sequences. Analyst, 133, 52-57.

Uludag,Y., Piletsky,S.A., Turner,A.P., Cooper,M.A., 2007. Piezoelectric sensors based on molecular imprinted polymers for detection of low molecular mass analytes. FEBS J., 274, 5471-5480.

Updike,S.J., Hicks,G.P., 1967. Reagentless substrate analysis with immobilized enzymes. Science, 158, 270-272.

Vera,A., Aris,A., Daura,X., Martinez,M.A., Villaverde,A., 2005. Engineering the E. coli beta-galactosidase for the screening of antiviral protease inhibitors. Biochem. Biophys. Res. Commun., 329, 453-456.

Vet,J.A., Majithia,A.R., Marras,S.A., Tyagi,S., Dube,S., Poiesz,B.J., Kramer,F.R., 1999. Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc. Natl. Acad. Sci. U. S. A, 96, 6394-6399.

Villaverde,A., 2003. Allosteric enzymes as biosensors for molecular diagnosis. FEBS Lett, 554, 169-172.

Voltersvik,P., Albrektsen,G., Ulvestad,E., Dyrhol-Riise,A.M., Sorensen,B., Asjo,B., 2003. Changes in immunoglobulin isotypes and immunoglobulin G (IgG) subclasses during highly active antiretroviral therapy: anti-p24 IgG1 closely parallels the biphasic decline in plasma viremia. J. Acquir. Immune. Defic. Syndr., 34, 358-367.

Wachter,R.M., 2007. The family of GFP-like proteins: Structure, Function, Photophysics and Biosensor Applications. Introduction and Perspective. Photochemistry and Photobiology, 82, 339-344.

Watkins,B.A., Buge,S., Aldrich,K., Davis,A.E., Robinson,J., Reitz,M.S., Jr., Robert-Guroff,M., 1996. Resistance of human immunodeficiency virus type 1 to neutralization by natural antisera occurs through single amino acid substitutions that cause changes in antibody binding at multiple sites. J. Virol., 70, 8431-8437.

Webb,M.R., 2007. Development of fluorescent biosensors for probing the function of motor proteins. Mol Biosyst, 3, 249-256.

Wei,X., Decker,J.M., Wang,S., Hui,H., Kappes,J.C., Wu,X., Salazar-Gonzalez,J.F., Salazar,M.G., Kilby,J.M., Saag,M.S., Komarova,N.L., Nowak,M.A., Hahn,B.H., Kwong,P.D., Shaw,G.M., 2003. Antibody neutralization and escape by HIV-1. Nature, 422, 307-312.

Yang,S., Veide,A., Enfors,S.O., 1995. Proteolysis of fusion proteins: stabilization and destabilization of staphylococcal protein A and Escherichia coli beta-galactosidase. Biotechnol Appl Biochem, 22 (Pt 2), 145-159.

Young,K.R., Teal,B.E., Brooks,Y., Green,T.D., Bower,J.F., Ross,T.M., 2004. Unique V3 loop sequence derived from the R2 strain of HIV-type 1 elicits broad neutralizing antibodies. AIDS Res. Hum. Retroviruses, 20, 1259-1268.

Zar,H.J., Latief,Z., Hughes,J., Hussey,G., 2002. Serum immunoglobulin E levels in human immunodeficiency virus-infected children with pneumonia. Pediatr. Allergy Immunol., 13, 328-333.

Zaytseva,N.V., Montagna,R.A., Baeumner,A.J., 2005. Microfluidic biosensor for the serotype-specific detection of dengue virus RNA. Anal. Chem., 77, 7520-7527.

Zhang,B., 2004. Design of FRET-based GFP probes for detection of protease inhibitors. Biochem. Biophys. Res. Commun., 323, 674-678.

Zhang,P.F., Bouma,P., Park,E.J., Margolick,J.B., Robinson,J.E., Zolla-Pazner,S., Flora,M.N., Quinnan,G.V., Jr., 2002. A variable region 3 (V3) mutation determines a global neutralization phenotype and CD4-independent infectivity of a human immunodeficiency virus type 1 envelope associated with a broadly cross-reactive, primary virus-

neutralizing antibody response. J. Virol., 76, 644-655.

Zhang,Z., Zhao,Q.X., Fu,J.L., Yao,J.X., He,Y., Jin,L., Wang,F.S., 2006. Characteristics of HIV-1-specific CD8 T-cell responses and their role in loss of viremia in children chronically infected with HIV-1 undergoing highly active antiretroviral therapy. Chin Med. J. (Engl.), 119, 1949-1957.

Zhou,X., Liu,L., Hu,M., Wang,L., Hu,J., 2002. Detection of Hepatitis C virus by piezoelectric biosensor. J. Pharm. Biomed. Anal., 27, 341-345.

8. ABBREVIATIONS

SPR: Surface Plasmon Resonance

IR: Infrared wave

HIV: Human Immunodeficiency Virus

FRET: Fluorescence Resonance Energy Transfer

HCV: Hepatitis C Virus

SARS: Severe Acute Respiratory Syndrome

ONPG: Ortho nitrophenil β-D-galactopyranoside

CPRG: Chlorophenol red β–D-galactopyranoside

FDG: Fluorescein β-D-galactopyranoside

X-gal: 5-bromo-4-chloro-3-indolil-β-D-galactopiranoside

AP: Alkaline Phosphatase

GFP: Green Fluorescent Protein

BLIP: β-lactamase inhibitory protein

DHFR: Dihydropholate reductase

EGFP: Enhanced green fluorescent protein

FMDV: Food-and-mouth disease virus

HA: Influenza hemaglutinin

HSV: Herpes simplex virus

LA: Lipid A

LF: Lethal factor

LPS: Lipopolysaccharide

MBP: Maltose binding protein

TEV: Tobacco etch virus

CTL: Citotoxic cell

MHC: Major Histocompatibility Complex

AIDS:	Acquired Immunodeficiency Sindrome
SIV:	Simian Immunodeficiency virus
IFN:	Interferon
WHO:	World Health Organization
ART:	Antiretroviral therapy
CD4BS:	CD4 binding site
FDA:	Food and Drug Administration
IgA, IgE, IgM, IgG:	Heavy chain isotypes. Immunoglobulins A, E, M and G
IgG1, IgG2, IgG3, IgG4:	Isotypes of immunoglobulin G
Km:	Michaelis-Menten constant
Kcat:	Catalytic constant
Kcat/Km:	Catalitic efficiency
ELISA:	Enzyme-linked immunosorbent assay
IPTG:	Isopropyl β-D-1-thiogalactopyranoside

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