

**USE OF CALIX[4]ARENES TO  
RECOVER THE SELF-ASSEMBLY  
ABILITY OF MUTATED p53  
TETRAMERIZATION DOMAINS**

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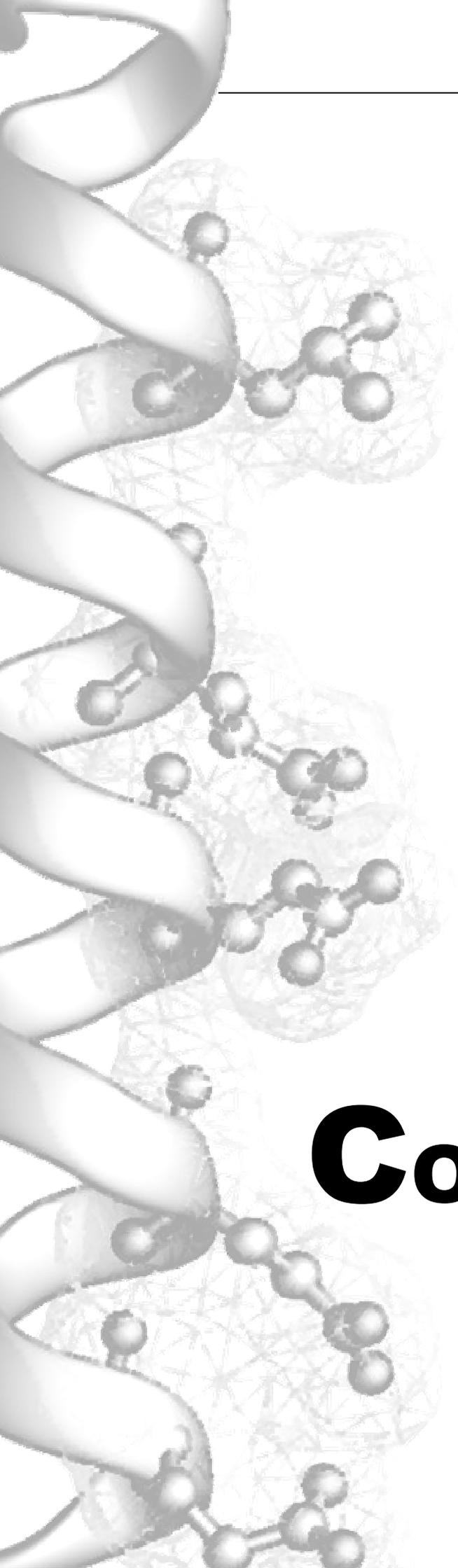
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# CONCLUSIONS



In view of the experimental results presented in the previous chapters, and considering the initial objectives of this thesis, the main conclusions reached comprise the following:

## Objective 1

- i. **Three natural mutated tetramerization domains of p53** with different assembly abilities have been successfully obtained. They are **G334V** (a missense point mutation found in lung cancers), **R337H** (an inherited point mutation associated to pediatric adrenocortical carcinoma) and **L344P** (an inherited point mutation associated to the Li-Fraumeni syndrome).

Mutants G334V and R337H were produced by site-directed mutagenesis of the former clone encoding the wild-type p53TD; they were expressed and purified using the protocol established for the native protein. In contrast, L344P required construction of a new clone and establishment of a new purification protocol based on His-tag.

It is worth mentioning that the use of auto-inducing media for the production of both non-labeled and isotopically labeled proteins improves the expression levels lowering costs and demanding less effort.

- ii. The **self-assembly properties** of the proteins have been evaluated in a battery of **biophysical experiments**.

**DSC** and **CD temperature-dependent** experiments have determined the **thermal stability** of the proteins. The mutant proteins are clearly less stable than the wild-type species, thus suggesting a less packed structure.

The pH-dependent stability of R337H was thoroughly studied and the results successfully reproduce those published by DiGiammarino *et al.*

On the contrary, amyloid aggregation of G334V could not be observed under the conditions reported by Higashimoto *et al.* This was probably due to the two additional, unfolded tails of the recombinant protein, which were not present in the synthetic tetramerization domain employed by those authors. Nevertheless, some kind of  $\beta$ -aggregation process was observed at high protein concentrations.

Circular dichroism has also provided information about the **oligomeric state** of each protein. At high protein concentrations, both G334V and R337H can display the **same CD profile** than the wild-type protein. This, in conjunction with the  **$^1\text{H}$ - $^{15}\text{N}$ -HSQC spectra** and the **chemical cross-linking** results, suggests similar levels of structure for these proteins.

As expected, mutant L344P is an unstructured monomeric polypeptide with rather low solubility.

Interestingly, despite the fact that mutant G334V is thermally more stable than R337H, at room temperature its tetramerization equilibrium is less shifted. Hence, linking thermal stability with tetramerization level at low temperatures is not always reliable.

Finally, preliminary **ESI-MS** experiments have detected the non-covalent tetramer for each of the structured proteins. This is the first ever report of this type of observation for p53TD.

## Objective 2

The concept that a synthetic ligand is able to stabilize the oligomeric state of a protein with defective assembly abilities has been successfully proven.

The case of the designed calix[4]arenes and the p53TD has provided further understanding of protein-ligand recognition events.

### i. Calix4bridge

Experimental evidence has certainly proved that calix4bridge, a tetraguanidinium-biscrown-calix[4]arene ligand, **specifically interacts** with p53 tetramerization domain as well as with the structured mutants G334V and R337H, and through this interaction **the tetrameric assembly** of the mutated proteins is **stabilized**.

From a **structural** point of view, the **design model** has been **confirmed** by NMR experiments on both the protein and the ligand. The ESI-MS results have been the conclusive evidence of the **1:2** tetramer-ligand **stoichiometry**.

**Mechanistic and thermodynamic information** about the binding event provided by NMR underscores the **role of protein flexibility** in the molecular recognition process. Whereas for the compact and stable wild-type protein the interaction proceeds via a lock-and-key model with two equivalent and independent binding sites ( $K_D \sim 280\mu\text{M}$ ), for R337H the two ligand molecules bind sequentially –and likely cooperatively– with much higher affinity ( $K_D < 50\mu\text{M}$ ). Conversely, for G334V, despite the fact that it has a less compact structure too, the affinity does not differ much from that of the wild-type protein, presumably because the mutation affects the architecture of the binding site and the calixarene may not fit properly.

The low affinity displayed by a control ligand with four amino groups instead of the guanidinium ones highlights the **crucial role of the carboxylate-guanidinium chelation** for the interaction between protein and ligand. In numbers, the affinity for the wild type protein is reduced nearly four times ( $K_D \sim 1\text{mM}$ ).

Taken together, these findings indicate that calix4bridge can only **stabilize** the tetrameric assemblies of already structured proteins; it can **not recover** those tetramerization domains with severe mutations in which even the secondary structure elements have been lost (*i.e.* L344P).

## ii. Calix4prop

Introduction of flexibility in the calix[4]arene molecule (*i.e.* calix4prop) have resulted in a **higher affinity ligand** for the tetramerization domain of p53wt and R337H, which largely **increases the thermal stability** for both proteins. Nevertheless, in the binding event the **structure** of the proteins is **seriously affected**.

Interestingly, mutant G334V becomes more prone to  $\beta$ -aggregation in the presence of calix4prop, phenomenon which remains to be understood.

Biophysical results by several techniques strongly suggest that the **sequential and cooperative binding** of **two** molecules of calix4prop to the protein may proceed with “perturbation” of the hydrophobic core of the native tetrameric structure, hypothetically by the direct interaction with the non-polar, conformationally-free propyl chains from the calixarene lower rim.

The thermodynamic dissociation constant for the complex p53TD-calix4prop has been estimated to be in the **low micromolar** range. **Protein flexibility** is essential for a **tighter interaction**. In turn, the evaluation of calix[4]propyl derivatives with different functional groups in the upper rim has highlighted the **relevance of an optimal guanidinium-carboxylate chelation** in the molecular recognition event.

Taken together, **flexibility** in both partners (*i.e.* the protein and the ligand), rather than penalize, seems to **favor** the interaction. Entropy and enthalpy penalties from loss of degrees of freedom and protein structural changes can be overcome by tighter hydrophobic and electrostatic protein-ligand interactions. The final balance of so many contributions can not be easily foreseen and results in unpredicted behaviors.

## In addition...

In addition to the goals initially proposed, and based on the results published by Ungaro and co-workers, other molecular recognition properties of the calix[4]arenes have also been studied.

Tetraguanidinium calix4bridge and calix4prop were found to **interact with DNA**, likely through the recognition of the phosphate moieties by the guanidinium groups. Calix4prop not only interacted with plasmidic DNA, but also **condensed** it into particles that could be **transfected into cells** (probably via endocytosis). Regrettably, calix4prop was **highly toxic**, thus limiting its utility for *in vivo* biological assays.

