PhD Thesis

European Joint Doctorate on Theoretical Chemistry and Computational
Modeling

**Quantum Mechanics/Molecular Mechanics
modeling of biological relevant reactions catalyzed
by enzymes**

Concepción Meliá Fortuño

Castelló, July 2017

Escola Superior de Tecnologia i Ciències Experimentals

Departament de Química Física i Analítica

Àrea de Química Física

Vicent Moliner Ibáñez, Profesor Catedrático del *Departament de Química Física i Analítica* de la Universitat Jaume I de Castellón y **Silvia Ferrer Castillo**, Personal Investigador del *Departament d'Enginyeria Mecànica i Construcció* de la Universitat Jaume I de Castellón,

CERTIFICAN:

Que el trabajo con título “*Quantum Mechanics/Molecular Mechanics (QM/MM) modeling of biological relevant reactions catalyzed by enzymes*” ha sido realizado por Concepción Meliá Fortuño bajo nuestra dirección, para optar al grado de doctora en Química.

Así, autorizan la presentación de este trabajo a efectos de seguir los trámites correspondientes de la Universitat Jaume I de Castellón.

Castellón de la Plana, a 30 de mayo de 2017.

Dr. Vicent Moliner Ibáñez

Dra. Silvia Ferrer Castillo

Director de Tesis

Directora de Tesis

A mis padres

A Alberto

A mis hijos

*“No se consigue la iluminación imaginándose figuras de luz,
sino haciendo consciente la oscuridad”*

CARL GUSTAV JUNG

Acknowledgements/Agradecimientos

En primer lugar agradecer esta Tesis Doctoral a mis directores de Tesis, Vicent Moliner y Silvia Ferrer, por la ayuda y confianza que me han brindado durante todo este tiempo.

Además quiero extender mi agradecimiento a toda la gente que ha participado de una u otra forma en el desarrollo de la Tesis:

Al Dr. Aurélien de la Lande por ser mi tutor durante la estancia en el *Laboratoire de Chimie-Physique de la Université Paris Sud CNRS, UMR 8000*.

Agradezco a Merche, la secretaria del departamento, por la ayuda que tan eficientemente me ha brindado en los trámites administrativos.

A los profesores y personal del departamento de Química Física y Analítica que me han asesorado tanto científica como burocráticamente.

Agradecer a mi familia, marido e hijos.

Finalmente quisiera agradecer a las fundaciones y organismos que han financiado mi investigación:

- Generalitat Valenciana (Proyecto PROMETEO/2009/053)
- FEDER y Ministerio de Economía y Competitividad Español (Proyecto CTQ2012-36253-C03)
- Universitat Jaume I (Proyecto P1 1B2011-23)

Abstract (English Version)

This PhD thesis reports theoretical studies of enzyme catalyzed reactions based on the use of hybrid Quantum Mechanics/Molecular Mechanics (QM/MM) potentials. It is divided in two blocks, one devoted to the study of the hydrolysis of a β -lactam antibiotic, and a second block devoted to exploring the mechanism of a copper monooxygenase.

Firstly, a theoretical study of the hydrolysis of a β -lactam antibiotic, Cefotaxime (CEF), was carried out in gas phase at different levels of theory. Later, the reaction was studied in solution, describing the sub-set of atoms of the QM region with semiempirical and density functional theory (DFT) methods while classical force fields were used to describe the explicit solvent water molecules. QM/MM Molecular Dynamics (MD) simulations were used to generate the potential of mean force (PMF) for the reaction in solution. The energy profiles showed a step-wise mechanism that is kinetically determined by the nucleophilic attack of a water molecule activated by the proton transfer to the carboxylate group of the substrate.

In the next step, the mechanism of hydrolysis of two antibiotics, CEF and Imipenem (IMI) were explored, in the active site of a mononuclear β -lactamase, CphA from *Aeromonas hydrophila*. The results showed significant differences between both antibiotics when analyzing the transition state (TS) structures. This study sheds some light into the origin of the selectivity of the different Metallo- β -lactamases (M β LS).

In the second block of the thesis, QM/MM methods have been applied to the study of an enzyme belonging to the family of copper monooxygenases which are known to perform dioxygen-dependent hydroxylation of aliphatic C-H bonds. In particular the mechanism of hydroxylation by peptidylglycine α -hydroxylating monooxygenase (PHM), with two uncoupled metal sites, was the subject of the study. Due to the electronic complexity of the system, *ab initio* MD simulations were required in order to get a proper description of the reaction including the intrinsic flexibility of the active site in the modeling protocol. The main observation was the extremely fast rebound step after the initial hydrogen abstraction step promoted by the cupric-superoxide adduct.

These studies, performed at several levels of theory and methods, illustrate basic features of reaction mechanisms used by enzymes to catalyze chemical reactions and, from the methodological point of view, the dependence of the quality of the results on the theoretical approach used to afford the proper analysis of these important chemical problems.

Abstract (Spanish Version)

Esta tesis de doctorado presenta estudios teóricos de reacciones catalizadas por enzimas basados en el uso de potenciales híbridos QM/MM. La tesis se divide en dos bloques, uno dedicado al estudio de la hidrólisis de un antibiótico β -lactámico, y un segundo bloque dedicado a la exploración del mecanismo de una monooxigenasa de cobre.

En primer lugar, se realizó un estudio teórico de la hidrólisis de un antibiótico β -lactámico, el Cefotaxime (CEF), en vacío a diferentes niveles de cálculo. A continuación la reacción se estudió en disolución acuosa utilizando métodos semiempíricos y métodos basados en la teoría del funcional de la densidad (DFT) para describir el subconjunto de átomos de la región QM, mientras que las moléculas de agua se describieron de forma discreta con campos de fuerza clásicos. Se utilizaron simulaciones de dinámica molecular (MD) con potenciales híbridos QM/MM para generar el potencial de fuerza media (PMF) para la reacción en disolución. Los perfiles energéticos mostraron un mecanismo por etapas que está determinado cinéticamente por el ataque nucleofílico de una molécula de agua activada por la transferencia de un protón al grupo carboxilato del sustrato.

En el siguiente paso, se exploró el mecanismo de hidrólisis de dos antibióticos, CEF e Imipenem (IMI) en el centro activo de una enzima β -lactamasa mononuclear, CphA de *Aeromonas hydrophila*. Los resultados mostraron diferencias significativas entre los dos antibióticos al analizar las estructuras del estado de transición (TS). Este estudio arroja cierta luz en el origen de la selectividad de las diferentes Metallo- β -lactamasas (M β LS).

En el segundo bloque de la tesis, se estudió una enzima de la familia de las monooxigenasas de cobre las cuales se sabe que realizan la hidroxilación dependiente de dioxígeno de los enlaces C-H alifáticos. En particular, el objeto de estudio fue el mecanismo de hidroxilación por la peptidilglicina α -monooxigenasa hidroxilante (PHM), que contiene dos centros metálicos no acoplados. Debido a la complejidad electrónica del sistema, fue necesario simulaciones de MD *ab initio* con el fin de obtener una descripción adecuada de la reacción incluyendo la flexibilidad intrínseca del centro activo. El resultado principal fue la descripción de la etapa inicial extremadamente rápida posterior al paso inicial de extracción de hidrógeno promovido por el aducto de superóxido cúprico.

Estos estudios, realizados a varios niveles de teoría con diferentes métodos, ilustran características básicas de los mecanismos utilizados por enzimas para catalizar reacciones, así como, desde el punto de vista metodológico, las dificultades de su modelización por la dependencia de la calidad de los resultados con el nivel de cálculo utilizado para obtener un análisis apropiado de estos importantes problemas químicos.

List of Contributions

The present Thesis is based on the following publications:

- *Computational Study on Hydrolysis of Cefotaxime in Gas Phase and in Aqueous Solution*. Conchín Meliá, Silvia Ferrer, Vicent Moliner, Iñaki Tuñón and Juan Bertrán. *Journal of Computational Chemistry*. 2012. 33, 1948-1959. DOI: 10.1002/jcc.23030.
The editors of *Journal of Computational Chemistry* invited us to use an image of this study as a cover of the volume 33, Issues 23-24, 2012.
- *Theoretical studies of the hydrolysis of antibiotics catalyzed by a metallo- β -lactamase*. Conchín Meliá, Silvia Ferrer, Vicent Moliner and Juan Bertran. *Archives of Biochemistry and Biophysics*. 2015. 582, 116-126. DOI: 10.1016/j.ab.2015.01.013.
- *Investigation of the Hydroxilation Mechanism of Noncoupled Copper Oxygenases by Ab Initio Molecular Dynamics Simulations*. Conchín Meliá, Silvia Ferrer, Jan Řezáč, Olivier Parisel, Olivia Reinaud, Vicent Moliner and Aurélien de la Lande. *Chemistry a European Journal*. 2013. 19, 17328-17337. DOI: 10.1002/chem.201301000.

Contribution report:

The author contributed in the papers doing the tasks of theoretical calculations, analysis of the results obtained, writing a first draft of the manuscript and reviewing the final version of the manuscript.

List of Acronyms and Abbreviations

AM1	Austin Model 1
B3LYP	Becke Three-parameter Lee-Yang-Parr functional
BIA	Biapenem
BOMB	Born-Oppenheimer Molecular Dynamics
CEF	Cefotaxime
DFT	Density Functional Theory
DβM	Dopamine- β -monooxygenase
E	Enzyme
ES	Enzyme-Substrate Complex
FEP	Free Energy Perturbation
GHO	Generalized Hybrid Orbital
IMI	Imipenem
IRC	Intrinsic Reaction Path
LBHB	Low Barrier Hydrogen Bonds
LRET	Long-Range Electron Transfer
LSCF	Local Self-Consistent Field
M06-2X	Truhlar-Zhao Minnesota 06 (0.54 exchange) functional
MC	Michaelis-Menten Complex
MD	Molecular Dynamics
MEP	Minimum Energy Path
MM	Molecular Mechanics
MβL	Metallo- β -Lactamase
NAC	Near Attack Conformations
NDDO	Neglect of Diatomic Differential Overlap
P	Product
PAL	Peptidyl- α -hydroxyglycine α -Amidating Lyase
PAM	Peptidylglycine α -Amidating Monooxygenase
PBC	Periodic Boundary Conditions
PDB	Protein Data Bank
PES	Potential Energy Surface
PHM	Peptidylglycine α -Hydroxylating Monooxygenase
PM3	Parametrized (NDDO) Model 3
PMF	Potential of Mean Force
QM	Quantum Mechanics
QM/MM	Quantum Mechanics/Molecular Mechanics
S	Substrate
SCF	Self Consistent Field
SEP	Superficie de Energía Potencial
SβL	Serine- β -Lactamase
TI	Thermodynamic Integration
TS	Transition State
TST	Transition State Theory
TβM	Tyramine- β -Monooxygenase
WHAM	Weighted Histogram Analysis

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1. Introduction and Objectives (English version)

1. Introduction and Objectives

The increasing use of antibiotics has induced the development of different resistance mechanisms in bacteria. β -Lactamases are the major cause of resistance of bacteria to β -lactam antibiotics. These enzymes cleave the amide bond of the β -lactam ring inactivating the antibiotic. The β -lactamases are classified into four groups. Class A, C and D utilize an active site serine in the catalysis of the β -lactam hydrolysis, whereas class B consists of metalloenzymes with one or two zinc cofactors. Although the catalytic mechanism of the serine-based enzymes is relatively well established, our understanding of class B β -lactamases is less developed. It is accepted that the mechanism of hydrolysis includes two steps; nucleophilic attack on the carbonyl group of the β -lactam ring and protonation of N atom with concomitant scission of the carbon-nitrogen bond. A question of debate is whether the bond scission and the protonation of the N take place concertedly or not. Zinc is an essential trace element and the second most abundant transition metal in biology. It participates in all major biochemical pathways and plays many important roles in the perpetuation of genetic material. The ability of the zinc atom to participate in strong but readily exchangeable ligand binding and the exceptional flexibility in the coordination geometry around the metal center allow the use of zinc in a variety of biological applications. Zinc exerts many biological effects through chemical reactions catalyzed by enzymes. The Zn(II) ion present in metalloenzymes can adopt four-, five-, or six-coordinate geometry because the energy barrier for the conversion between various geometries is relatively small. Metallo- β -lactamases have very broad substrate spectra. Despite much effort, no clinically effective inhibitor has been found. These enzymes represent a potent threat to β -lactam antibiotics.

The first part of the present PhD Thesis is focused in the theoretical study of the hydrolysis of β -lactam antibiotics in gas phase and in aqueous solution (see Publication 1 in Chapter 10). In this first contribution the β -lactam ring hydrolysis of Cefotaxime (CEF), which belongs to the group of Cephalosporines, was studied in gas phase including the full molecule and a water molecule as a nucleophilic agent, in order to illustrate the basic features of the reaction mechanism and the dependence on the theoretical approach. Studies in gas phase can render important information of the required level of theory to properly describe a specific kind of chemical transformation, to explore the features of a substrate or to select the proper distinguished reaction coordinate to be used in more complex calculations including the environment effects. The PESs for the hydrolysis of CEF in gas phase were obtained at PM3, B3LYP/6-31G* and M06-2X/6-31G* levels of theory. PM3 and M06-2X rendered a molecular mechanism in three steps, while the B3LYP functional described the reaction in two steps. The reaction mechanism was also studied in aqueous solution by exploring QM/MM PESs and PMFs. PESs were used to state the mechanism, followed by the generation of the corresponding PMFs for every single step of the reaction. The hydrolysis of CEF in aqueous solution can proceed by two different

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molecular mechanisms, stepwise and concerted. The rate limiting step of stepwise mechanism, the first step of the full process, presented lower barrier than the one of the concerted mechanism, thus suggesting which is the most favorable reaction path.

Based on this first study, the second contribution of the present PhD Thesis, corresponds to the reaction mechanisms studied in the active site of a mononuclear β -lactamase (see Publication 2 in Chapter 10). In particular, the hydrolysis of Imipenem (IMI), an antibiotic belonging to the subgroup of carbapenems, and the hydrolysis of Cefotaxime (CEF), a third-generation cephalosporin antibiotic, were studied in the active site of CphA from *A. hydrophila*. Keeping in mind that CphA shows only activity against carbapenem antibiotic, a comparative analysis of the results obtained for both inhibitors can shed some light into the origin of the selectivity of the different MBLs. In this case the mechanism of hydrolysis of the β -lactam ring took place by means of a concerted mechanism. The QM region was described by means of the PM3 semiempirical method, using zinc parameters optimized for metalloenzymes. The Zinc ion present in the enzyme presented different coordination sphere depending on the antibiotic. In the IMI reactants structure zinc was hexacoordinated, while the reactive structure with the CEF had a tetrahedral geometry. These results could explain the different reactivity of the studied antibiotics.

The second part of the present PhD Thesis reports a study of the mechanism of hydroxylation by a copper monooxygenase (PHM). Many peptides, such as hormones, growth factors, and neurotransmitters, require α -amidation of their carboxy terminus to be fully biologically active. The α -amidation is carried out in the trans-Golgi network and in secretory granules of neural and endocrine tissues by two sequential reactions. The first reaction is the stereospecific hydroxylation of the C α of a C-terminal peptidylglycine, a reaction catalyzed by PHM. The second reaction, an N-dealkylation that yields the amidated peptide and glyoxylate, is catalyzed by peptidyl- α -hydroxyglycine α -amidating lyase (PAL). Although the two enzymes can be expressed and can function independently, in most species they are expressed as a bifunctional single-chain enzyme, peptidylglycine α -amidating monooxygenase (PAM). Despite the intense efforts focused on the elucidation of the PHM activity, several aspects of the catalyzed peptide amidation mechanism remain unclear. The reduction of molecular oxygen into the hydroxylated product requires the electron transfer from a copper site acting as electron reservoir, namely Cu_H, to that binding O₂ and the substrate, namely Cu_M.

The theoretical studies on this second block of the PhD Thesis were focused in the hydroxylation of noncoupled copper oxygenases (see Publication 3 in Chapter 10). In particular, the molecular mechanism of the peptidylglycine α -hydroxylating monooxygenase (PHM) catalytic cycle was

1. Introduction and objectives

explored by hybrid DFT/MM Born-Oppenheimer molecular dynamics (BOMD) simulations. The reaction was explored from the binding of dioxygen to the Cu_M active site followed by the attack of the C-H bond in the substrate and further evolution steps. The BOMD methodology involves propagating the motion of the nuclei on the PES by application of Newton's laws. The forces acting on the nuclei are computed on-the-fly at the DFT/MM level. As a complement to geometry optimizations, this computationally intensive approach permits the inclusion of the intrinsic dynamics of the Cu_M active site in the modeling strategy. It is possible to assess the stability of the optimized structures or to explore the conformational landscape visited by the system on the picosecond timescale. The MD simulations have turned out to be crucial for revealing a rebound step following the initial hydrogen abstraction in PHM. Simulations of a long-range electron transfer (LRET) from the Cu_H active site also suggested that this was the final element necessary to complete the reaction. Overall, the results provided an updated view of the PHM catalytic cycle that is fully consistent with the large body of available experimental data.

The present PhD Thesis is concluded with a summary of the conclusions and future perspectives that can be deduced from the reported results.

1.1. Introduction and Objectives (Spanish version)

El uso creciente de antibióticos ha inducido el desarrollo de diferentes mecanismos de resistencia en las bacterias. Las β -lactamasas son la principal causa de resistencia de las bacterias a los antibióticos β -lactámicos. Estas enzimas rompen el enlace amida del anillo β -lactámico, inactivando el antibiótico. Las β -lactamasas se clasifican en cuatro grupos. Las clases A, C y D utilizan una serina en el centro activo en la catálisis de la hidrólisis del anillo β -lactámico, mientras que la clase B consiste en metaloenzimas con uno o dos cofactores de zinc. Aunque el mecanismo catalítico de las enzimas basadas en serina está relativamente bien establecido, nuestra comprensión de las β -lactamasas de clase B está menos desarrollada. Se considera como muy probable que el mecanismo de hidrólisis incluye dos etapas; el ataque nucleofílico sobre el grupo carbonilo del anillo β -lactámico y la protonación del átomo de N con rotura concomitante del enlace carbono-nitrógeno. Sin embargo, no existe consenso sobre si la ruptura de los enlaces y la protonación del N se llevan a cabo de forma concertada o por etapas. El zinc es un oligoelemento esencial y el segundo metal de transición más abundante en biología. Participa en todas las principales vías bioquímicas y desempeña muchos papeles importantes en la perpetuación del material genético. La capacidad del átomo de zinc para participar en la unión de ligando es alta pero es un catión fácilmente intercambiable y la excepcional flexibilidad en la geometría de coordinación alrededor del centro del metal justifica que el zinc participe en una gran variedad de sistemas biológicos. El zinc ejerce muchos efectos biológicos a través de reacciones químicas catalizadas por enzimas. El ion Zn (II) presente en las metaloenzimas puede adoptar geometrías de cuatro, cinco o seis coordenadas porque la barrera de energía para la conversión entre las diversas geometrías es relativamente pequeña. Las metalo- β -lactamasas tienen un espectro de sustrato muy amplio. A pesar de mucho esfuerzo, no se ha encontrado ningún inhibidor clínicamente efectivo y, en definitiva, estas enzimas representan una potente amenaza para el uso médico de los antibióticos β -lactámicos.

La primera parte de la presente tesis doctoral se centra en el estudio teórico de la hidrólisis de antibióticos β -lactámicos en fase gaseosa y en disolución acuosa (véase la publicación 1 en el capítulo 10). En esta primera contribución se estudió la hidrólisis del anillo β -lactámico de Cefotaxima (CEF), perteneciente al grupo de Cefalosporinas, en fase gaseosa, incluyendo la molécula completa y una molécula de agua como agente nucleófilo, para ilustrar las características básicas del mecanismo de reacción y la dependencia del enfoque teórico utilizado. Los estudios en fase gaseosa pueden proporcionar información importante del nivel requerido de teoría para describir apropiadamente un tipo específico de transformación química, explorar las características de un sustrato o seleccionar la coordinada de reacción distinguida apropiada que se usará en cálculos más complejos incluyendo los efectos ambientales. Las PES para la hidrólisis de CEF en fase

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gas se obtuvieron con diferentes Hamiltonianos PM3, B3LYP/6-31G* y M06-2X/6-31G*. Los resultados con PM3 y M06-2X describieron un mecanismo molecular en tres etapas, mientras que el funcional B3LYP describió la reacción en dos etapas. A partir de estos resultados, la reacción se estudió en disolución acuosa mediante la exploración de las PESs y los PMFs con potenciales híbridos QM/MM. Las PESs se usaron para obtener el mecanismo, seguido por la generación de las PMF correspondientes para cada paso de la reacción. La hidrólisis de CEF en disolución acuosa puede proceder por medio de dos mecanismos moleculares diferentes, por pasos y concertado. El paso limitante del mecanismo por pasos, el primer paso, presentó una menor barrera energética que el mecanismo concertado, sugiriendo que es éste el mecanismo más probable.

A partir de este primer estudio, la segunda contribución de la presente tesis doctoral, corresponde a los estudios de los mecanismos de reacción en el centro activo de una β -lactamasa mononuclear (véase la publicación 2 en el capítulo 10). En particular, se estudió la hidrólisis de Imipenem (IMI), un antibiótico perteneciente al subgrupo de carbapenemes, y la hidrólisis de cefotaxima (CEF), un antibiótico cefalosporina de tercera generación, en el centro activo de CphA de *A. hydrophila*. Teniendo en cuenta que CphA sólo muestra actividad contra el antibiótico carbapenem, un análisis comparativo de los resultados obtenidos para ambos inhibidores puede arrojar alguna luz sobre el origen de la selectividad de los diferentes M β LS. En este caso, el mecanismo de hidrólisis del anillo β -lactámico tuvo lugar por medio de un mecanismo concertado. La región QM fue descrita por medio del método semiempírico PM3, usando los parámetros del Zinc para metaloenzimas. El ion Zinc, presentó diferente coordinación según sea el antibiótico objeto de estudio. En la estructura de reactivos con IMI el zinc estaba hexacoordinado, mientras que la estructura de reactivos con CEF tuvo una geometría tetraédrica. Estos resultados podrían sugerir el origen de la selectividad de estos enzimas por el sustrato.

La segunda parte de la presente tesis doctoral reporta un estudio del mecanismo de hidroxilación por una enzima monooxigenasa de cobre (PHM). Muchos péptidos, tales como hormonas, factores de crecimiento y neurotransmisores, requieren que la α -amidación de su grupo carboxílico terminal sea biológicamente activa. La α -amidación se lleva a cabo en la red trans-Golgi y en gránulos secretorios de tejidos neurales y endocrinos mediante dos reacciones secuenciales. La primera reacción es la hidroxilación estereoespecífica del C α de una peptidilglicina C-terminal, una reacción catalizada por PHM. La segunda reacción, una desalquilación en N que produce el péptido amidado y el glioxilato, es catalizada por el alfa-amidación de litio (PAL) de peptidil- α -hidroxiglicina. Aunque las dos enzimas pueden expresarse y pueden funcionar independientemente, en la mayoría de las especies se expresan como una enzima monocatenaria bifuncional, la mono-oxigenasa α -amidante de peptidilglicina (PAM). A pesar de los intensos

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esfuerzos enfocados en la elucidación de la actividad de PHM, varios aspectos del mecanismo de amidación de péptido catalizado siguen siendo incógnita. Por ejemplo, la reducción del oxígeno molecular en el producto hidroxilado requiere la transferencia de electrones desde un centro de cobre que actúa como depósito de electrones, a saber Cu_H , a ese O_2 de unión y al sustrato, a saber Cu_M .

Los estudios teóricos sobre este segundo bloque de la presente tesis doctoral se centran en la hidroxilación de oxigenasas de cobre no acopladas (véase la publicación 3 en el capítulo 10). En particular, se exploró el mecanismo molecular del ciclo catalítico de monohidroxilación α -hidroxilante (PHM) de peptidilglicina mediante simulaciones DFT/MM híbridas mediante dinámicas moleculares Born-Oppenheimer (BOMD). Se estudió desde la unión de dioxígeno al centro activo de Cu_M seguido del ataque del enlace C-H en el sustrato, así como etapas de evolución adicionales. La metodología BOMD implica propagar el movimiento de los núcleos en la PES mediante la aplicación de la ecuación de Newton. Las fuerzas que actúan sobre los núcleos se calculan sobre la marcha al nivel DFT/MM. Como complemento a las optimizaciones de geometría, este enfoque computacional permite incluir la dinámica intrínseca del centro activo Cu_M . Así es posible evaluar la estabilidad de las estructuras optimizadas o explorar el paisaje conformacional visitado por el sistema en la escala de tiempo del picosegundo. Las simulaciones de MD resultaron ser cruciales para revelar un paso de rebote que sigue a la abstracción inicial del hidrógeno en la PHM. Las simulaciones de una transferencia de electrones de largo alcance (LRET) del centro activo de Cu_H sugirieron también que éste es el elemento final necesario para completar la reacción. En general, los resultados proporcionaron una visión actualizada del ciclo catalítico PHM compatible con la gran cantidad de datos experimentales disponibles.

La presente tesis se concluye con un resumen de las conclusiones y perspectivas futuras que se pueden deducir de los resultados presentados.

2. Enzymatic catalysis

2. Enzymatic Catalysis

Enzymes are generally proteins that lead, control and speed up the chemical reactions in biological systems.^[1] In fact, this control can be carried out not only catalyzing the main reaction, but inhibiting the side reactions that can take place between the reactants or between the reactants and the solvent.^[1]

An enzyme is a protein molecule built up of 20 different species of amino acids linked together to form one or more long chains. Its molecular weight ranges from ten thousand to hundreds of thousands of Daltons.^[2] The long chain(s) of which they are constituted must be folded in such a way that a three-dimensional pocket or cleft is created into which the compounds attacked (known as substrates) fit in a very precise way.^[2] This part of the enzyme where the substrate binds to and where the catalysis occurs is the *active site*.^[3,4]

Some enzymes require cofactors to be active, which are non-proteic components and which may be classified as metal ions^[4] metal ions, such as Mg^{2+} , Zn^{2+} , Mn^{2+} , Cu^{2+} , etc.; or organic, as the molecules named coenzymes. The latter, at the same time, can be co-substrates, which are only transiently associated with the enzyme (NAD^+ , NADP^+ , etc.), or prosthetic groups, which are permanently bounded to the enzyme, often by covalent bonds (heme group).

Summarizing, the inactive apoenzyme binds with the cofactor to form a holoenzyme, which is already active.

We should bear in mind that, in order to complete the catalytic cycle, the enzyme must return to its original state.

The most remarkable properties of enzymes as catalysts are: ^[4,5]

- **Catalytic power:** The rate of a non-enzymatic reaction varies from 10^{-7} to 10^{-1} s^{-1} , while in an enzymatic reaction it can be in a range from 10 to 10^7 s^{-1} .^[6]
- **Specificity:** Enzymes have a vast degree of specificity with respect to their substrates and products. It also means that rarely have side products.
- **Milder reaction conditions:** Enzymes usually work under neutral pH, mild temperature and atmospheric pressure, fact that makes them a very unique and outstanding kind of catalysts.
- **Capacity for regulation:** The activity of some enzymes can vary in response to concentration of substances other than their substrates.

It is important to highlight that the purpose in mechanistic enzymology is to determine the exact reaction mechanisms and the physicochemical phenomena contributing to the enormous rates reached by enzymes.^[1,6]

2. Enzymatic Catalysis

Since nineteenth century, scientists have tried to understand the enzymatic behavior. In 1835, Berzelius developed the first theory about chemical catalysis, where he pointed out that a malt extract known as diastase (nowadays, it is proved that it contains α -amylase) catalyzed the hydrolysis of the starch more efficiently than sulphuric acid.^[4]

Later on, about 1850, Pasteur suggested that the fermentation process was only possible in alive cells (the theory of the “vital power”).^[4] Nevertheless, in 1897, Buchner demonstrated that the enzyme activity could be expressed without the need of the cell. Specifically, he extracted the enzymes which catalyze the alcoholic fermentation in the yeast cells.

The word enzyme was first used by Kühne in 1878, and it means “in yeast”,^[3] what intended to emphasize that the catalytic activity was the manifestation of an extract or secretion rather than of the whole organism.

In 1894 Fischer proposed, the *lock and key* model,^[7] which stated that both the enzyme and substrate's structures are complementary and rigid (see Figure 1).

Figure 1. Lock and key model.

In 1913, Leonor Michaelis and Maud Menten,^[8] suggested a new model based on the following catalytic scheme (for a single-substrate enzyme-catalyzed reaction):^[5]

First of all, the enzyme-substrate complex (ES), known as the Michaelis-Menten's coordination complex, is formed in a reversible way. Then, this ES complex breaks down in a slow step to give products (P), and the enzyme is released (E).

If we apply the steady-state approximation to ES , which means that the concentration of ES remains constant because the rate of its formation equals the rate of its breakdown, then:

2. Enzymatic Catalysis

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_{cat}[ES] = 0 \quad (2)$$

$$[ES] = \frac{k_1[E][S]}{k_{-1} + k_{cat}} \quad (3)$$

The system is in a steady state in which the rate of formation of ES is exactly balanced by the rate of its removal and that the substrate is in vast molar excess over the enzyme.^[2]

The concentration of free enzyme, $[E]$, is given by the total amount of enzyme present, minus the amount present as the ES complex:

$$[E]_{total} = [E] + [ES] \quad (4)$$

$$[E] = [E]_{total} - [ES] \quad (5)$$

$[E]_{total}$ equals to the concentration of enzyme present at the start of the reaction, as the enzyme is not consumed during the reaction.

If equation (5) is replaced in equation (3):

$$[ES] = \frac{k_1([E]_{total} - [ES])[S]}{k_{-1} + k_{cat}} \quad (6)$$

$$[ES] = \frac{[E]_{total}[S]}{\frac{k_{-1} + k_{cat}}{k_1} + [S]} \quad (7)$$

Therefore, the rate of the reaction (taking into account the irreversible step) is:

$$v_{cat} = k_{cat}[ES] = \frac{k_{cat}[E]_{total}[S]}{K_M + [S]} \quad (8)$$

where,

$$K_M = \frac{k_{-1} + k_{cat}}{k_1} \quad (9)$$

is the Michaelis-Menten constant, and characterizes the thermodynamics of the formation of the ES complex. K_M is the concentration of substrate at which the reaction rate is half of the maximum rate. It is independent of the total amount of E and it gives a numerical value to the affinity of the enzyme (the higher the K_M is, the lower the affinity of the enzyme for its substrate is).^[2]

2. Enzymatic Catalysis

The reaction rate is usually rewritten as:

$$v_{cat} = \frac{v_{max} [S]}{K_M + [S]} \quad (10)$$

known as the Michaelis-Menten equation (reaction rate for a one-substrate reaction catalyzed enzymatically).

A special feature of the enzymatic reactions is the behavior of the rate against the concentration of substrate:

When $[S] \ll K_M$, the rate depends almost linearly on $[S]$, and $[S]$ is rate limiting

When $[S] \gg K_M$, the enzyme reaches a saturation limit by the substrate (a fact not observed in non-enzymatic reactions), so, $[E]$ is rate limiting

$$[S] \uparrow \uparrow \rightarrow v_{max} = k_{cat} [E]_{total} \quad (11)$$

because $K_M \ll [S]$, v_{max} is the rate at saturating levels of substrate. It defines the situation where $[S]$ is so high that almost the whole enzyme is present in the form of ES .

Figure 2. Rate of a catalyzed reaction against the concentration of substrate. Figure adapted from reference^[2].

For the steady-state approximation, K_M can be interpreted as a measure of the lifetime of ES complex, the ratio of the rate of breakdown by any route to the rate formation.

From equation (11), we obtain the catalytic constant:

$$k_{cat} = \frac{v_{max}}{[E]_{total}} \quad (12)$$

2. Enzymatic Catalysis

Is the first-order rate constant of the decomposition of the enzyme complex to products, but may be more complex if the formation of the products from the complex involves several steps.

In 1926, Sumner isolated an enzyme in its crystal form for the first time, and he also proved that these crystals were constituted by protein.

In 1930, Haldane proposed the induced-fit hypothesis^[9]: the active site of the enzyme adopts an optimal conformation to interact with the substrate, but only when the substrate itself is present (see Figure 3).

Figure 3. Induced fit model. Figure adapted from references^[2,10].

Pauling,^[11] in 1946, proposed that the enzymes catalyze reactions by preferentially binding the transition state (TS). An enzyme may bind to the TS of the reaction that catalyzes with greater affinity than to its substrates or products. The active site is complementary to the TS, not to the substrate in its fundamental state.

In Figure 4 we observe the energetic profile of a catalyzed and non-catalyzed reaction; the former would correspond to the kinetic scheme of equation (1).

Figure 4. Free energy diagram of a reaction catalyzed by an enzyme (red line) compared to the non-catalyzed reaction (blue line). Figure adapted from reference [12].

2. Enzymatic Catalysis

The non-catalyzed reaction occurs in just one step, with an activation free energy of $\Delta G_{nocat}^{\ddagger}$. In contrast, the reaction catalyzed by the enzyme, in this example, consists of two steps: first, the enzyme-substrate, known as the Michaelis-Menten complex (MC), is formed, losing binding energy, ΔG_{bind}^{MC} . Secondly, the chemical reaction takes place itself, with an energetic cost of $\Delta G_{cat}^{\ddagger}$. This value is smaller than $\Delta G_{nocat}^{\ddagger}$ and it can be related to the reaction rate, k_{cat} , by the Transition State Theory (TST).

The catalytic power of an enzyme is usually expressed by the ratio k_{cat}/k_{nocat} , that is, it is proportional to the difference between the activation free energy of the uncatalyzed reaction and of the catalyzed one.

As it can be observed in Figure 4:

$$-\Delta G_{bind}^{TS} + \Delta G_{cat}^{\ddagger} = -\Delta G_{bind}^{MC} + \Delta G_{nocat}^{\ddagger} \quad (13)$$

$$\Delta G_{nocat}^{\ddagger} - \Delta G_{cat}^{\ddagger} = \Delta G_{bind}^{MC} - \Delta G_{bind}^{TS} \quad (14)$$

If the enzyme speeds up the reaction, the left term of equation (14) should be positive, so ΔG_{bind}^{TS} should be greater, in its absolute value, than ΔG_{bind}^{MC} (it must be kept in mind that binding energies are always negative in value). This argument supports the theories that assume that the catalytic role of the enzymes lies in the *stabilization of the TS versus the MC*.^[12-15]

In conclusion, we can classify the most popular theories about enzyme catalysis into two groups: *TS-theories* and *MC-theories*, this is, transition state stabilization and substrate preorganization, respectively.

- *Electrostatic effects*^[5,13-17]: Belongs to the group of *TS-Theories*. In the site of an enzyme, the dipoles are rigidly held in a fixed orientation, pointing toward the substrate. However, in solution, large electrostatic attraction does not arise because any force created by moving opposite charges toward each other is balanced by reorientation of the solvent dipoles (see Figure 5). It seems that enzyme can stabilize in a more effective way polar transition states and other charge distributions.

2. Enzymatic Catalysis

Figure 5. a) Solvation in water; b) pre-oriented dipoles in an enzymatic environment. Figure adapted from reference^[15].

- *Near attack conformations (NAC)*^[6,18]: It is the clearest proposal of the *MC-theories*. Near attack conformations (NACs) are structures with the reacting groups very close and oriented so as the reaction proceeds overcoming a small energy barrier. The preorganization of the active site of the enzymes in order to promote the formation of NACs would be a critical component of enzyme catalysis.

A note of caution here about terminology: substrate preorganization is not to be confused with enzyme preorganization.^[12]

Apart for the main theories proposed to explore the origin of the enzyme catalysis, other factors have been proposed to contribute to the catalytic power of enzymes, as follows:

- *Low Barrier Hydrogen Bonds (LBHB)*^[19,20]: Weak hydrogen bonds are electrostatic attractive interactions between partially positively charged protons of donors and electronegative atoms with, at least, one non-bonding electron pair (usually nitrogen or oxygen). The energy of this type of hydrogen bonds is about 2-8 Kcal per mole. Whereas there is another type of hydrogen bonds, named low barrier hydrogen bonds (LBHB), which are shorter (less than 2.5 Å), very strong and they are formed in transition states or in metastable intermediates, stabilizing them. Its formation can supply 10 to 20 kcal per mole, what can bring enough energy to overcome difficult reactions.

2. Enzymatic Catalysis

Figure 6. Energy diagrams of a weak and strong LBHB. The zero point vibrational energy of the proton is depicted in dotted lines. X and Y are heteroatoms. Figure adapted from reference^[6].

In Figure 6, we can see the difference between both hydrogen bonds. In the weak hydrogen bonds, the energy wells of the interacting heteroatoms are quite separated and there is a high energy barrier in between. As the zero point energy of the proton is lower than the barrier, the proton would be covalently bonded to X and electrostatically attracted to Y. On the other hand, in the strong hydrogen bonds, the picture is different: the heteroatoms are close enough that the barrier is almost as low as the zero point energy of the proton, taking place a significant covalent bonding of the proton with both X and Y. It is necessary for the LBHB to be formed that both heteroatoms show similar proton affinities.

- *Strain or distortion*^[21]: The enzyme can induce strain or distortion in the bond of the substrate involved in the reaction, which can make the cleavage of this bond to occur easily, what means that the enzyme favours the formation of the Michaelis complex.
- *Desolvation*^[22]: The adsorption of the substrate in the active site of the enzyme can take place only if all the molecules of solvent are squeezed out from between both of them.
- *Orbital steering*^[10,16,23]: One of the main functions of the active site is to help in the orbital steering. Thus, the orbitals of the substrate and the orbitals of the catalytic group of the enzyme line up and lead the enzyme-substrate complex to the transition state.
- *Entropic factors*^[16,24]: A naïve explanation of entropy would be a measure of the degree of randomness or disorder of a system. So, the more disordered, the higher the entropy.

In solution, the reactions are slow because the rapprochement of the reactants implies a loss in entropy, whereas in enzymatic reactions, there is no loss of rotational or translational entropy from reactants to transition state as the reactants are already close (in the active site). This advantage in entropy is paid for the enzyme-substrate binding

2. Enzymatic Catalysis

energy; the rotational and translational entropies of the substrate are lost in the formation of the enzyme-substrate complex, and not during the chemical steps.

- *Tunneling effect*^[6]: Hydrogen tunneling is possible in any enzymatic reaction in which a light particle atom is transferred. It is a quantum mechanical effect in which molecules with energy lower than the barrier can overcome it and react, increasing the reaction rate.
- *Dynamic effects*^[25-43]: There is heated debate about the role of protein dynamics in enzyme catalysis, especially for reactions that involve transfer of hydrogen (H^+ , H , H^-), in which quantum tunneling is significant. It has been suggested that “promoting protein motions”, i.e., specific fluctuations that might reduce the barrier height or promote tunneling by reducing donor-acceptor distances, can drive enzymatic reactions. Such models include promoting vibrations, environmentally coupled tunneling, and vibrationally enhanced ground-state tunneling. Several of these proposals suggest that the anomalous temperature and pressure dependences of experimentally observed reaction rates and kinetic isotope effects are consequence of protein motions on the pico- to femtosecond timescale that reduce the width and/ or height of the potential energy barrier along the chemical reaction coordinate. However, a connection between promoting motions and potential energy barrier modulation has never been demonstrated directly, and recent work has shown that the temperature dependence of kinetic isotope effects can be accounted for by conformational effects for a number of enzymes. Whereas some authors postulate dynamics as a key driving force in catalysis, others have performed analyses showing activation free-energy reduction, which is an equilibrium property, to be source of catalysis. Enzyme reactions, and particularly their dynamics, present formidable challenges for study, and progress requires a combination of theoretical, experimental, and computational approaches. A powerful tool to probe the nature of enzyme motions and their response to changes in the reaction conditions is the study of ‘enzyme isotope effects’.^[43-55] The coupling of protein motions to enzyme catalysis is revealed as a difference between the kinetic properties of the isotopologous enzymes, because mass-dependent translational, vibrational and rotational motions are altered by heavy isotope substitution, whereas the potential energy surface and electrostatic properties are unaffected.^[45,46]
- *Covalent catalysis*^[4]: It suggests that the increase in the reaction rate is due to the temporary formation of a covalent bond between the enzyme and the substrate.

2. Enzymatic Catalysis

- *Acid-based catalysis*^[4]: It can be a general acid, base or both catalysis. The general acid catalysis is a process in which the transfer of a proton from an acidic species diminishes the free energy of the transition state of a reaction. A reaction can be also simulated by means of general basic catalysis, where a base abstracts a proton in a partial way.
- *Metal ion catalysis*^[4]: Almost one third of the known enzymes need metal ions to work. These enzymes can be metalloenzymes, which contain metal ions stronglybonded (Fe^{2+} , Fe^{3+} , Cu^{2+} , Zn^{2+} , etc.); or enzymes activated by a metal, which weakly fix metal ions already present in solution (Na^+ , K^+ , Mg^{2+} , Ca^{2+}).

Keeping in mind the amount of hypotheses and the factors suggested as responsible of the origin of enzymatic catalysis, it is obvious that the understanding of these biological catalysis is not an easy task. Therefore, the exploration of the enzyme reaction mechanisms, at an atomic-level, is required to shed some light on these complex systems. Their understanding can have important consequences in science and technology.

For each type of enzyme there will probably be some factors that are more relevant than others in terms of their catalytic activity, or simply the origin of the catalytic power of the enzymes would lie in specific combination of several of these factors. In this Thesis these theories are applied to two different enzymes: Metallo- β -lactamases and Noncoupled Copper Oxygenases, in order to observe the different factors that influence the catalytic power of them.

3. Metallo- β -Lactamases

3. Metallo- β -Lactamases

The M β LS family was defined in 1997 as a new superfamily of metallohydrolases.^[56] There has been a growing concern on this zinc-dependent β -lactamases since, despite catalyzing the same reaction, it seems that S β LS and M β LS do not share any structural nor mechanistic similarity^[57] and, in fact, the latter are unaffected by all clinically useful inhibitors of the serine-active enzymes.^[58] In fact, no M β L inhibitors are available for clinical use.^[59]

Three subgroups of M β L have been further identified depending on sequence structure and activity similarities. B1 and B3 subclasses possess a binuclear active site, which requires one or two Zn(II) ions for full activity and are able to hydrolyze carbapenems, penicillins and cephalosporins.^[59] B2 subclass, unlike those from subclasses B1 and B3, are fully active with one zinc ion bound and possess a narrow spectrum of activity, hydrolyzing carbapenem substrates almost exclusively.^[60] Initially, a reduced number of structures of B2 M β L, CphA from *Aeromonas hydrophila*,^[61] ImiS from *Aeromonas veronii* bv. Sobria^[62] and Sfh.I from *Serratia fonticola*,^[60] have been crystallized, being the CphA the most studied one. In particular, three different structures, two of them in the apo form and the last one corresponding to the N220G mutant in complex with a biapenem (BIA) derivative, were obtained.

Figure 7: CphA structure from *Aeromonas hydrophila*
adapted from PDB file with code 1X8I.

The CphA-Bia complex structure has shown how the zinc metal accommodates in the Zn₂ site, with a trigonal bipyramidal coordination formed by Asp120, Cys221, His263, the carboxylate oxygen and the N4 atoms of Bia. Based on these X-ray structures, Garau et al.^[61] suggested a mechanism involving a non-metal-binding water nucleophile, activated by His118, that would attack the carbonyl carbon of the substrate, leading to

3. Metallo- β -Lactamases

cleavage of the C7-N4 bond of the lactam ring. This proposal has been supported by QM/MM theoretical calculations of Xu et al.^[63,64] although suggesting that Asp120 would be the base activating the water molecule, instead of His118. In a more recent paper, Wu et al.^[65] proposed a complete reaction mechanism for the hydrolysis of biapenem antibiotic catalyzed by CphA, arguing that the CphA-Bia complex determined by Garau et al. would belong to a minor pathway, in contrast to the original suggestion. In this regard, simulations performed by Gatti^[66] suggest that the bicyclic derivative of Garau et al. would not be formed inside the enzyme active site. Hydrolyzed biapenem might be released first, cyclization would bind back to the active site.

Based on QM/MM calculations, an alternative mechanism was proposed by Simona et al.^[67,68] where the nucleophilic attack and the proton transfer to the nitrogen atom of the lactam ring would occur in a single concerted step. According to this proposal, the mechanism requires the activation of a second catalytic water molecule in the active site of the enzyme. This mechanism would be in agreement with experimental studies of Sharma et al.^[62] based on proton inventories showing that at least one proton transfer must be involved in the rate limiting step. Nevertheless, the proposal is based on the existence of a conformation of the MC in which the substrate binds the zinc metal through a water molecule. This model is not confirmed by the structural studies of Crowder et al.^[69] based on enzyme-product complexes, that suggests a direct contact between the zinc metal and the carboxylate of the substrate. An initial structure presenting this direct contact was used by Xu et al.^[63-65] to propose a step-wise mechanism that renders an estimated free energy barrier for the nucleophilic attack of ca. 14 kcal/mol,^[63] a value in very good agreement with the kinetic experiments of Garau et al. Nevertheless, this comparison requires the hypothesis that such step was the rate limiting step of the enzymatic cycle, apparently in contradiction with the proton inventory experiments of Crowder et al.^[69] and with QM/MM computationally exploration of the full mechanism performed by Simona et al.^[67] In particular, the second step related with the proton transfer from Asp120 to nitrogen atom of substrate, would become the rate limiting step, with a total free energy barrier of ca. 24 Kcal/mol.

4. β -lactam antibiotics

4. β -Lactam antibiotics

β -lactam antibiotics are the most effective chemotherapeutic agents for the treatment of bacterial infections,^[69,70] accounting for more than half of the world's antibiotic market. The mechanism of the antibacterial activity of β -lactams involves the inhibition of the biosynthesis of the bacterial cell wall peptidoglycan. Nevertheless, despite much progress in antibiotics design has been done during the past decades, the increasing use of these compounds has induced the development of different resistance mechanism in pathogenic microorganisms.^[71] One of the strategies evolved by bacteria to defend themselves from antibiotics is the production of β -lactamases,^[72] which hydrolyze the cyclic four membered ring to biologically inactive the drug (See Scheme 1).

Scheme 1. Hydrolysis of β -lactam ring.

Today, more than 500 β -lactamases are known, classified into four groups,^[73] A-D, according to their amino acid sequence.^[74] Groups A, C and D, also called serine- β -lactamases (S β LS), utilize an active site serine as a nucleophile,^[71] while B group required 1 or 2 Zn(II) ions to perform the hydrolysis and thus are called metallo- β -lactamases (M β LS).^[75] There has been a growing concern on this zinc-dependent β -lactamases since, despite catalyzing the same reaction, it seems that S β LS and M β LS do not share any structural nor mechanistic similarity^[57] and, in fact, the latter are unaffected by all clinically useful inhibitors of the serine-active enzymes.^[58] Thus, a detailed knowledge of the hydrolysis of the four membered ring of β -lactam antibiotics reaction mechanism is required in order to know the possible ways of inhibiting bacteria activity. It is accepted that hydrolysis includes two steps; nucleophilic attack on the carbonyl group of the β -lactam ring and protonation of N atom with scission of the carbon-nitrogen bond. In this regard, there is still a question of debate on the timing of carbon-nitrogen scission bond and the protonation of the N, which can even take place concertedly.

4. β -Lactam antibiotics

Figure 8. Structure of cephalosporin places where modifications can be made.

As example of antibiotic, it has been chosen for this PhD Thesis the so called Cefotaxime (CEF), which belongs to the group of the Cephalosporins. This is the second major group of β -lactam antibiotics to be discovered. The structure of cephalosporin (Figure 5) is a bicyclic system containing a four membered β -lactam ring. The β -lactam ring is fused to a six-membered dihydrothiazine ring. The larger ring relieves the strain in the bicyclic system to some extent, but it is still a reactive system. Many analogues of cephalosporin have been made which demonstrate the importance of the β -lactam ring within the bicyclic system, the ionized carboxylate group at position 4, and the acylamino side chain at position 7. In fact, there is a limited number of places where analogues present modifications are shaded in orange as depicted in Figure 8,^[76] variations of the 7-acylamino side chain, variations of the 3-acetoxymethyl side chain and extra substitution at carbon 7.

Figure 9. Structure of Cefotaxime.

The reaction mechanism of the antibiotic Cefotaxime in condensed media is the focus of the present study, the goal is to understand the reaction in solution, and further, to compare with the studies in the enzyme. To carry out the computation in condensed media, we use hybrid quantum mechanics/molecular mechanics methods explained in next section.

Although penicillins and cephalosporines are the best known and most researched β -lactams, there are other β -lactam structures which are of

4. β -Lactam antibiotics

great interest in the antibacterial field. An example is the group of carbapenems.

Carbapenems show a high resistance to β -lactamases. This resistance has been ascribed to the presence of hydroxyethyl side chain. (see Figure 10) The stereochemistry of the side chain at substituent 6 is opposite from the usual stereochemistry in penicillins, another factor in the resistance of this agent to β -lactamases.

Figure 10. Structure of Carbapenem places where modifications can be made.

One example of carbapenem antibiotic is Imipenem (IMI). IMI is useful in treating some infections which do not respond to cephalosporines, or in treating infections which have become resistant to the conventional β -lactams.^[76]

Figure 11. Structure of IMI.

The investigation of this PhD Thesis explores the mechanisms in the active site of a mono-nuclear β -Lactamase. In particular, we are studying the hydrolysis of Imipenem (IMI), and antibiotic belonging to the subgroup of a carbapenems, and the Cefotaxime (CEF), a third-generation cephalosporin antibiotic, in the active site of CphA from *Aeromonas hydrophila* (see Figure 7). Keeping in mind that CphA show only activity against carbapenem antibiotic, a comparative analysis of the results obtained for both inhibitors will be used to shed some light into the origin of the selectivity of different M β L.

5. Noncoupled Copper Oxygenases

5. Noncoupled Copper Oxygenases

In Nature, the family of noncoupled copper monooxygenases comprising peptidylglycine α -hydroxylating monooxygenase (PHM), dopamine β -monooxygenase (D β M), and tyramine β -monooxygenase (T β M) provides a fascinating source of inspiration.^[77-79] These ascorbate- and dioxygen-dependent copper active sites, customarily referred to as Cu_M and Cu_H (see Scheme 3 for the reaction catalyzed by PHM).^[80] The two active sites are separated by a solvent-filled cleft with a width of around 10 Å and orchestrate the transfer of the four electrons and two protons required for substrate hydroxylation with concomitant release of H₂O.

Scheme 2. Reaction catalyzed by PHM.

The imposing mass of biochemical data accumulated on these enzymes over the last three decades suggest that PHM, D β M, and T β M probably share a common mechanism with the chemistry taking place at the Cu_M site and the Cu_H site serving as an electron reservoir.^[80] Although the identification of the C-H bond-activating process is still debated,^[77,81,82] converging biochemical arguments support an enzymatic mechanism initiated by a hydrogen-abstraction step promoted by a cupric-superoxo ([Cu_M^{II}(O₂^{••})]⁺) adduct.^[82-85] Mononuclear biomimetic cupric-superoxo complexes have been characterized by a few groups,^[86-88] and some studies have also explored their oxidative behavior.^[89-96] These investigations on biomimetic systems thus provide support for a mechanism involving a hydrogen-abstraction step in the enzymes. On the other hand, the nature of steps following the hydrogen abstraction is still highly debated and conflicting proposals have been advanced for the enzymatic mechanism. Computational approaches can advantageously complement experimental studies on enzymatic systems. In recent years, modeling based on quantum chemistry has, for example, confirmed the oxidative properties of cupric-superoxo complexes that were built as models of the enzymatic Cu_M active site^[82,97-99] or as models of biomimetic systems.^[100-102]

5. Noncoupled Copper Oxygenases

Figure 12: PHM structure adapted from PDB file with code 1SDW.

Kamachi and Yoshizawa and co-workers^[98,99] were the first to successfully apply a density functional theory/molecular mechanics (DFT/MM) scheme to determine the equilibrium structures of the cupric-superoxo ($[\text{Cu}_M^{\text{II}}(\text{O}_2^{\cdot\cdot})]^+$) adducts within D β M and to determine the energy profile associated with hydrogen abstraction from the dopamine molecule. Crespo et al.^[82] shortly after reported a similar study with PHM. Both studies concluded that reaction pathways proceeding through a cupric-superoxo complex were unlikely and as an alternative proposed that copper-oxo ($[\text{Cu}_M\text{O}]^{2+}$)^[82] or copper-oxil ($[\text{Cu}_M^{\text{II}}\text{O}^{\cdot\cdot}]^+$) species was responsible for the hydrogen abstraction step. Such conclusions appear contradictory with experimental studies on PHM and D β M supporting the copper-superoxo ($[\text{Cu}_M^{\text{II}}(\text{O}_2^{\cdot\cdot})]^+$) hypothesis.^[77,84]

The nature of the steps following the hydrogen abstraction is still highly debated and conflicting proposals have been advanced for the enzymatic mechanism. Hybrid QM/MM approaches have acquired an important role in the investigation of enzymatic mechanisms by computer simulations because they provide an efficient means to including the environment of the enzymatic active sites.^[103,104] So, in this PhD Thesis it is reported an investigation of the mechanism of hydroxylation by PHM by using hybrid quantum/classical potentials. *Ab initio* MD simulations have been carried out to properly describe the complex electronic process but also in order to include the intrinsic flexibility of the active sites in the modeling protocol.

6. Computational Methods

6.1. Quantum Mechanics Methods

Semiempirical methods and DFT-based calculations were used to perform the theoretical studies of this PhD Thesis.

The semiempirical methods used are the AM1^[22] and the PM3^[105,106], both come from the Neglect of Diatomic Differential Overlap (NDDO) model which relaxes de constraints on the two center two electron integrals. The AM1 method has the ability to reproduce hydrogen bonds and better estimates of activation energies for reactions. PM3 method limited to Gaussian functions per atom instead of the four in AM1 and includes several parameters to carry out semiempirical calculations in molecular modeling.

The DFT methods used in this PhD Thesis are B3LYP^[107] and M06-2X^[108]. The DFT methodology uses the electron density and introduces the correlation. The B3LYP functional uses the semiempirical combination of exact exchange and gradient correction performs significantly better and fits experimental atomization energies with an impressively small average. The M06-2X is a hybrid meta exchange-correlation functional, it is a high-nonlocality functional with double amount of nonlocal exchange, and it is parameterized only for nonmetals.

6.2. Hybrid QM/MM Methods

The inclusion of the environment effects in the computational simulations is basic when we are studying enzymatic reactions. For this reason, the protein environment effects should be included in the computational simulations, because the specific interactions they generate can modify the biochemical process. The most useful solution lies in describing the region where the reaction takes place by means of QM methods, while the MM methods are chosen to treat the surrounding amino acids of the protein environment, and/or the solvent molecules.^[109] The resulting scheme of the combination of both techniques is referred to as the hybrid quantum mechanics/molecular mechanics method (QM/MM). This methodology allows large-scale biomolecular systems involving a chemical reaction to be simulated, with a reasonable computational cost and with an acceptable degree of accuracy. The QM/MM methods were first proposed by Warshel and Levitt in 1976.^[103] It combines the accuracy of the quantum mechanical methods with the low computational cost of the molecular mechanical methods.

In 2013, Martin Karplus, Michael Levitt, and Arieh Warshel were awarded with the Nobel Prize in Chemistry for the “development of multiscale models for complex chemical systems”. The investigations that they conducted in the 1970s on chemical computational models. These researchers combined the principles of quantum theory and classical physics to reproduce the chemical processes that occur in nature.

First of all, and in order to use this hybrid methodology, the QM and the MM regions of the system should be selected (see Figure 13).

Figure 13. Different regions of a QM/MM system.

The QM region should contain, at least, the atoms which participate directly in the formation or breaking of the covalent bonds. Usually, it includes the substrate, the cofactor (if it exists) and the amino acids of the active site of the enzyme which are involved in the reaction (i.e., they make covalent bonds or electron density transfer with the substrate or cofactor)

6.2. Hybrid QM/MM Methods

The MM region contains the rest of the atoms in the system, i.e., the rest of the amino acids of the enzyme and the solvent molecules or counterions.

Finally, the boundary conditions are a collection of restrictions applied to the system due to the fact that it is impossible to simulate an infinite system, as it will be explained later on.

To be able to compute the energy and the forces of each atom in the system, an effective Hamiltonian, $\hat{H}_{eff}$, is constructed and the time independent Schrödinger equation is solved for the electronic wavefunction, ψ_{el} , of the atoms in the QM part:

$$\hat{H}_{eff} \psi(r, R_\alpha, R_M) = E(R_\alpha, R_M) \psi(r, R_\alpha, R_M) \quad (15)$$

Ψ depends on the electrons' coordinates (r), and on the position of both the quantum and classical nuclei (R_α and R_M , respectively).

This effective Hamiltonian in QM/MM methods is based on an additive scheme,^[110] This general classification is applied to schemes for which the QM and MM energies are considered complementary, the total energy of the system is obtained by adding them together and applying coupling terms or corrections.

$$\hat{H}_{eff} = \hat{H}_{QM} + \hat{H}_{MM} + \hat{H}_{QM/MM} + \hat{H}_{BC} \quad (16)$$

$\hat{H}_{QM}$ will depend on the quantum method used. It describes the electrons, nuclei and interactions among the atoms of the QM part and corresponds to an electronic Hamiltonian of the subsystem in vacuum,

$$\hat{H}_{QM} = -\frac{1}{2} \sum_i \nabla_i^2 + \sum_{ij} \frac{1}{r_{ij}} - \sum_{i\alpha} \frac{Z_\alpha}{r_{i\alpha}} + \sum_{\alpha\beta} \frac{Z_\alpha Z_\beta}{R_{\alpha\beta}} \quad (17)$$

Equation (17) is the sum of the kinetic energy of the electrons, the electron-electron repulsion, the nucleus-electron attraction and the nucleus-nucleus repulsion.

- i, j : electronic coordinates
- α, β : nuclear coordinates
- r : electron-electron or nucleus-electron distance
- R : nucleus-nucleus distance
- Z : nuclear charge
- ∇ : kinetic energy operator or Laplacian operator

$\hat{H}_{MM}$ describes the interactions between the atoms described by means of molecular mechanics, as it only depends on the MM atoms.

$\hat{H}_{QM/MM}$ describes the interactions between the QM and MM atoms. Due to the fact that the MM atoms are represented by punctual charges and Van der Waals parameters, this Hamiltonian is expressed as:

$$\hat{H}_{QM/MM} = -\sum_{iM} \frac{q_M}{r_{iM}} + \sum_{\alpha M} \frac{Z_{\alpha} q_M}{R_{\alpha M}} + \sum \left\{ \frac{A_{\alpha M}}{R_{\alpha M}^{12}} - \frac{B_{\alpha M}}{R_{\alpha M}^6} \right\} \quad (18)$$

- i : the electrons of the QM atoms
- α : nuclei of the QM atoms
- M : refers to the MM atoms.

The first term of the equation (18) represents the electrostatic interactions between the MM atoms and the electrons of the QM part. The second term is the electrostatic interactions between the MM atoms and the nuclei of the QM atoms. The last term describes the Lennard-Jones interactions between the MM and QM atoms, otherwise the QM atoms would collide.

In this equation, just the first term is an operator as it contains the electrons' coordinates, so, it must be incorporated in the self consistent field (SCF) procedure. The remaining terms are constant for a given set of atomic coordinates, like the MM energy.

As we have already pointed out, $\hat{H}_{BC}$ appears due to the attempt to define something that is infinite (as, for example, a solvent) using a system than can only be defined in an explicit way, with a finite number of molecules.

- Cut-off Methods:

The principal problem with the non-bonding energy is the long-range electrostatic interaction. To perform calculations we must establish a cut-off distance beyond which the non-bonding interactions are no longer taken into account. There are different functions describing cut-off strategies to calculate non-bonding interactions.

Truncated function: This function avoids all the interactions beyond the cut-off distance (r_c). Each term in the non-bonding energy expression is multiplied by the function:

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$$S(r) = \begin{cases} 1 & r \leq r_c \\ 0 & r > r_c \end{cases} \quad (19)$$

In spite of its simplicity, the energy and its derivatives are no longer continuous, therefore, this option is not the most convenient.

Smoothing function: This method tappers the interaction continuously to zero at given distance. Now, the first derivative of the energy is continuous, but the second derivative is still not continuous.

There are two types of smoothing functions:

Switch function: It computes all the interactions inside a radius (r_{on}), from which it is smoothed to zero at the outer cut-off (r_{off}).

$$S(r) = \begin{cases} 1 & r \leq r_{on} \\ \frac{(r_{off}^2 - r^2)^2 (r_{off}^2 + 2r^2 - 3r_{on}^2)}{(r_{off}^2 - r_{on}^2)^3} & r_{on} < r \leq r_{off} \\ 0 & r > r_{off} \end{cases} \quad (20)$$

Shift function: It modifies the form of the interaction throughout its entire range.

$$S(r) = \begin{cases} \left[1 - \left(\frac{r}{r_c} \right)^2 \right]^2 & r \leq r_c \\ 0 & r > r_c \end{cases} \quad (21)$$

- Periodic Boundary Conditions (PBC)

The system is introduced in a solvent box which is replicated in an infinite way in the three dimensions of the space. Obviously, the finite system should have a regular shape in order to fill the space when it is replicated, what is achieved using very regular boxes such as cubic, orthorhombic or hexagonal ones.

In any case, when we are calculating the non-bonding energy, we should avoid that each particle could interact with its nearest copy in the system (this is the *minimum image convention*, see Figure 14). This fact makes the method cheaper and easier to implement, but it means that the cut-off distance for truncation of the non-bonding interactions must be less than or equal to half the length of the side of the periodic box (see Figure 14).

Figure 14. Schematic representation of the “minimum image convention”. Figure adapted from reference ^[111].

- Frontier bonds

In the study of very large systems, it is often necessary to split the system between different regions. In most of the systems under study, a covalent bond has to be cut when dividing the MM and QM part, so, a particular molecule must be divided in the QM and the MM part. That is why there is a need to do approximations in order to satisfy the valences of the QM atoms.

Link atoms: The more straightforward treatment of the frontier bonds is the *link atom*,^[103,112] which replaces the MM part of the bond by an atom, usually a hydrogen atom (due to the fact that the C and H electronegativities are similar). This link atom is invisible to the MM region and it is used to saturate the valences of the truncated bond.

Figure 15. Representation of the link atom methodology. The hydrogen in blue is the link atom.

Usually, the selected boundary atom is non-polar to allow a correct separation of the two electrons of the bond: one will stay in the QM region and the other will be removed in the MM region. So, the most common option to place the link atom is in the Csp^3 - Csp^3 bonds.

In practice, an instability source of the link atoms lies on the huge interaction observed between the wavefunctions of the regions close to the link atoms and to the MM atoms with partial charges. Therefore, the

6.2. Hybrid QM/MM Methods

following theory is introduced, which divides the system in three parts: QM region, MM region and an auxiliary region there are nuclei with normal nuclear charges but with some electronic density in some of the basis functions. So, the new Hamiltonian of the system is:

$$\hat{H}_{complete} = \hat{H}_{QM} + \hat{H}_{MM} + \hat{H}_{aux} + \hat{H}_{QM/aux} + \hat{H}_{QM/MM} + \hat{H}_{MM/aux} \quad (22)$$

- $\hat{H}_{aux}$: classical term which describes the electrostatic interaction of the frozen density and its nuclei with themselves
- $\hat{H}_{MM/aux}$: the interaction of the frozen density and its nuclei with the MM point charges and the non-bonded Lennard-Jones terms between the two regions
- $\hat{H}_{QM/aux}$: quite similar to $\hat{H}_{QM/MM}$, except that it adds two electron integrals with the orbitals one electron being frozen.

An alternative approach is to constrain the SCF solution to reflect the influence of the bonds that have been omitted.^[110]

Based on this, two other methods to treat the frontier bonds come out: the *Local self-consistent field (LSCF)*^[113,114] and the *Generalized Hybrid Orbital (GHO)*^[115]

Local Self-Consistent Field: In the LSCF method, the auxiliary region consists of a single frozen orbital on each QM boundary atom. The orbitals that describe the bonds QM atom-boundary atom are not optimized in the SCF cycle, they remain frozen but they can polarize the QM portion of the system because they act as frozen charge densities.

This method is very expensive as it needs a previous parametrization in each case.

Figure 16. LSCF methodology representation. X is the last QM atom.

6. Computational Methods

Generalized Hybrid Orbital: In the GHO method, the auxiliary region is bigger. It is similar to the link atom methodology because it uses a fully optimized molecular orbital to represent each frontier bond, but in this case, the orbital is surrounded by a charged environment which is much more realistic.

In this method, hybrid atomic orbitals are used as basis functions on the boundary atoms of the MM fragment. These hybrid orbitals are divided into auxiliary and active orbitals, the latter of which are optimized along with all other atomic orbitals of the QM fragment in the SCF calculations. Consequently, the chemical bond connecting the QM and the MM fragments is explicitly treated without introducing spurious “link atoms”. This method doesn’t need a previous parametrization in each case, but it can be used just for Csp^3 atoms, and it could be described as a particular case of the LSCF method.

Figure 17. GHO methodology representation. X is the last QM atom.

6.3. Potential energy surfaces

The generation of the potential energy surfaces (PES) are usually based on the Born-Oppenheimer approximation.

A PES is a representation of the potential energy (electronic and nuclear) depending on the several coordinates of the chemical system. Obviously, the choice of these coordinates depends on the nature of the chemical process that is being studied.

There are two main concepts that we need to describe prior the study of the PES: the gradient and the Hessian.

The gradient is a vector composed of the first derivatives of the potential energy with respect to the positions of the atoms.

$$\vec{g}_i = \frac{\partial U}{\partial \vec{r}_i} \quad \forall \quad i = 1, \dots, N \quad (23)$$

being N the number of atoms.

The Hessian is a symmetric square matrix which has as elements the second derivatives (or force constants) of the energy with respect to the displacement of two coordinates for all the atoms.

$$H_{ij} = \frac{\partial^2 U}{\partial \vec{r}_i \partial \vec{r}_j} \quad (24)$$

As it can be observed, equation (24) is a $3N \times 3N$ matrix which is symmetric due to the fact that $\partial^2 U / \partial x_i \partial y_j = \partial^2 U / \partial y_j \partial x_i$ (if the particles are the same, $i = j$). When the Hessian is diagonalized, $|H - \lambda I| = 0$, the eigenvalues equation can be solved:

$$F \vec{u}_i = \lambda \vec{u} \quad (25)$$

- F : is the diagonalized Hessian matrix
- $\vec{u}_i$: is the vector that represents the curvature principal axes and represents the normal modes
- λ : are the eigenvalues. There will be $3N$ eigenvalues and eigenvectors, being N the number of atoms in the system.

6.3. Potential Energy Surfaces

A PES is a function of the positions of all the atoms in the system. If the system has just two atoms, it is easy to visualize that the PES will depend on just one geometrical variable, the bond distance between the two atoms. So, the PES will be a bi-dimensional plot of the energy of the system versus this distance. However, if we consider a three-atom system, there will be three independent geometrical parameters, either three distances or two distances and an angle. This will lead to a lot more of calculations compared to the bi-dimensional PES and to another more important problem: we cannot represent four-dimensional plots as we live in a three-dimensional world. That is why there is a need to choose *distinguished reaction coordinates*, ξ_i , which are parameters that change in a substantial way during the reaction. For example, in this reaction:

a possible distinguished reaction coordinate would be the AB distance, which varies from infinite to a bond distance. Nevertheless, the BC distance would also have an important role from the transition state to products. So, the best option would be using both distances in a two-dimensional grid, or of to generate a one-dimensional potential energy profiles an antisymmetric combination. In any case, it should be pointed out that some experience is needed to choose the best distinguished reaction coordinate in each case.

Once the distinguished reaction coordinate or coordinates are chosen, each point of the PES will be computed varying (but keeping frozen in each calculation) this or these coordinates and minimizing the rest of the coordinates. In Figure 18, there is an example of a PES.

Figure 18. PES for $A + BC \rightarrow AB + C$ reaction, where R= reactants, TS= transition structure and P= products. (a) Contour plot, (b) three-dimensional plot.

6. Computational Methods

In a PES, as it can be observed in Figure 19, there are stationary points which are characterized by a gradient equal to zero, and they can be divided as minima and first-order saddle points.

When there is a minima and the Hessian matrix at this point is diagonalized, all the eigenvalues obtained are positive. This means that an infinitesimal displacement of the geometry of the system along the direction defined by any of its eigenvectors, will lead to an increase in energy. The minima are the reactants, products and intermediates.

Whereas, when there is a first-order saddle point and the Hessian matrix is diagonalized, all the eigenvectors obtained are positive but one. In this case, if we perform an infinitesimal displacement along the eigenvector corresponding to the negative eigenvalue, the energy will decrease. However, if the displacement is along the eigenvector associated to a positive eigenvalue, the energy will increase.

The eigenvector associated to the negative eigenvalue is known as the *transition vector* and indicates the direction from the transition structure to reactants and products.

Another important issue in a PES is the *intrinsic reaction path (IRC)*,^[116] the path that, departing from the transition structure, leads to the reactants and products, following the direction of the gradient. The initial point the transition structure, has a zero gradient, so, the initial direction of the IRC should be previously specified by the transition vector.

In a first approximation, we can obtain the IRC as the solution of:

$$d\vec{x} = \frac{\vec{g}(\vec{x})}{|\vec{g}(\vec{x})|} ds \quad (27)$$

where s is the path length from the transition structure or the reaction coordinate, and it is computed by:

$$(ds)^2 = \sum_i [(dx_i)^2 + (dy_i)^2 + (dz_i)^2] \quad (28)$$

It should be also pointed out that there are other methods, such as Page-McIver,^[117] that, in addition, uses the Hessian to compute the IRC.

But the problem is that the reaction path obtained depends on the coordinates used. Therefore, in 1970, Fukui^[118,119] demonstrated that if the mass-weighted Cartesian coordinates are used:

$$x_i = x_i \sqrt{m_i} \quad (29)$$

6.3. Potential Energy Surfaces

the requirement of following the gradient coincides with the path that would follow the system if, starting from the transition structure, it would go to reactants and products following a zero kinetic energy trajectory, i.e., a very slow trajectory. Therefore when we talk about an IRC, we really talk about reaction path which results from the solution of the equation (24) expressed in mass-weighted Cartesian coordinates.^[119]

There is another approximation to the IRCs, the MEPs or *minimum energy paths*,^[120] which connect reactants to products, passing through the transition state, along the minimum energy path, as its name already suggests.

The computation of the IRCs is very useful and necessary for a chemist because it establishes in an unequivocal way which reactants and products are connected to a located transition structure. Once the IRC is finished, in both directions of the transition vector, it should be checked that the system has reached the two minima, therefore, the point should be located and characterized as minima connected of the TS.

There are a wide variety of methods to locate minima and first order saddle points, and they are classified by the type of derivatives that they use:^[111]

- ***No derivatives or zero order methods***: They make use of energy values only (i.e., simplex method)
- ***First derivatives or first order methods***: They use values of the energy and its first derivatives (i.e., steepest descent or conjugate gradient).
- ***Quadratic or second order methods***: On the one hand, they can make use of the energy, its first derivative and they approximate its second derivatives^[121] (i.e., quasi-Newton, reduced basis-set Newton or truncated Newton algorithms). On the other hand, they can use energy values and its first and second derivatives^[121] (i.e., exact Newton or Newton-Raphson methods).

As it can be observed, there is a wide range of methods to locate stationary point structures, and the general rule to select one is using exact or quasi-exact Newton methods if the system is small because these methods are more efficient. However, if the system is large, first derivatives or, at the most, approximate second derivatives algorithms should be used.

On the other hand, when locating transition structures, it is more difficult as the energy should be minimized in N-1 directions and maximized in the others. It is almost exclusively achieved by Newton-Raphson methods (Baker or other eigenvector following methods).

6.4. Molecular dynamics

The information obtained in the study of chemical reactions in condensed media is not enough to describe the entire system in a representative manner.

The energy profile of a reaction in condensed media can be schematically represented as:

Figure 19. Schematic energy diagram of a reaction in a condensed media.

As it can be observed in Figure 20, in a reaction taking place in condensed media, there are many reactant structures, transition structures and product structures. This is due to the effect of the size of the system, especially when the environment is introduced explicitly: there is a wide range of accessible structures which are minima or saddle points in the PES, with similar geometries and energies. Therefore, the properties of the transition state will be the average properties of all the transition structures, and the same will occur with the reactant and product states.

It is also shown that, if we just pick up one reactant structure and one transition structure, we will obtain an energetic barrier ($\Delta E_1^\ddagger$) that can be different if we select other structures for reactants and transition structure ($\Delta E_2^\ddagger$), even though these latter structure provide almost the same chemical information. Moreover, the same argument can be used for the reaction energies. Consequently, the isolated study of the PESs is not enough, and

6.4. Molecular Dynamics

evaluation of as much configurations as possible is required to obtain average properties, which will be comparable to experimental data. Thus, it is necessary statistical simulations by means of *Molecular Dynamics* (MD) or *Monte Carlo* simulations (MC), which allow the exploration of many different conformations existing in the system. In our case the former has been selected the *Molecular Dynamics*.

The classical Hamiltonian of the system can be described as^[111]:

$$\hat{H}(\vec{p}_i, \vec{r}_i) = \sum_{i=1}^N \frac{1}{2m_i} \vec{p}_i^2 + V(\vec{r}_i) \quad (30)$$

- $\hat{H}$: the sum of the kinetic and potential energy terms and it is a function of the 3N particle momenta and the 3N particle positions
- $\vec{p}_i$: the momentum of a particle

V : the potential energy (computed by means of quantum methods, molecular mechanical methods or QM/MM methods). On the other hand, the Hamilton equations are:

$$\dot{\vec{r}}_i = \frac{\partial \hat{H}}{\partial \vec{p}_i} = \frac{\partial V}{\partial \vec{p}_i} = \vec{F}_i \quad (31)$$

$$\dot{\vec{p}}_i = -\frac{\partial \hat{H}}{\partial \vec{r}_i} = -\frac{\partial V}{\partial \vec{r}_i} = -\vec{F}_i \quad (32)$$

If we substitute equation (32) in equation (31), we obtain Newton's equation of motion for a particle:

$$\vec{F}_i = m_i \vec{a}_i \quad (33)$$

So, to perform a MD simulation, equation (33) (or equations (31) and (32)) must be solved for each particle in the system. As stated before, these equations account for classical particles. This is not a problem in the case of QM atoms due to the fact that the Born-Oppenheimer approximation allows treating separately the movement of the nuclei and the electrons: electron relaxation is almost instantaneous for a nuclear change. Thus, from a practical point of view, the main difference arises from how forces are calculated: using a force field for MM atoms, or by means of an electronic Hamiltonian in the case of the QM atoms.

Unfortunately, this approach has some drawbacks. Since the movement of all the nuclei is classical, it does not account for quantum effects

6. Computational Methods

which are important, for example, in the treatment of the dynamics of light particles (where quantum mechanical tunnelling effect can appear) and it neither include zero-point motion (the vibrational motion that all quantum mechanical systems undergo even at zero Kelvin).

Finally, in order to solve these classical equations, there are several algorithms, and the Verlet algorithm^[122] stands out from them.

If a time t , $\vec{r}(t)$ represents the positions of the atoms in the system, then the positions of these atoms at a time $t + \Delta t$, can be obtained from a Taylor expansion:

$$\vec{r}_i(t + \Delta t) = \vec{r}_i(t) + \Delta t \vec{v}_i(t) + \frac{\Delta t^2}{2} \vec{a}_i(t) + O(\Delta t^3) \quad (34)$$

- $\vec{v}_i(t)$: the velocity
- $\vec{a}_i(t)$: the acceleration at time t .

Similarly, the position of these atoms at a time $t - \Delta t$, is:

$$\vec{r}_i(t - \Delta t) = \vec{r}_i(t) - \Delta t \vec{v}_i(t) + \frac{\Delta t^2}{2} \vec{a}_i(t) - O(\Delta t^3) \quad (35)$$

Adding the equation (34) and (35), and taking into account equation (33):

$$\begin{aligned} \vec{r}_i(t + \Delta t) &= 2\vec{r}_i(t) - \vec{r}_i(t - \Delta t) + \Delta t^2 \vec{a}_i(t) \cong \\ &\cong 2\vec{r}_i(t) - \vec{r}_i(t - \Delta t) + \frac{\Delta t^2 \vec{F}_i(t)}{m_i} \end{aligned} \quad (36)$$

This equation describes the positions of the particles at a time $t + \Delta t$.

Subtracting equations (34) and (35):

$$\vec{v}_i(t) \cong \frac{1}{2\Delta t} (\vec{r}_i(t + \Delta t) - \vec{r}_i(t - \Delta t)) \quad (37)$$

This equation describes the velocities of the particles at a time t .

With both equations ((36) and (37)) it would be enough to integrate the equations of motion but, as we can observe, the velocities at a time t can only be computed once the positions at a time $t + \Delta t$ have been obtained, so, at the start of the simulation (*when $t=0$*) a different formula is needed.

6.4. Molecular Dynamics

To overcome this problem, the *velocity Verlet algorithm* appears,^[123] which use these formula:

$$\vec{r}_i(t + \Delta t) = \vec{r}_i(t) + \Delta t \vec{v}_i(t) + \frac{\Delta t^2}{2m_i} \vec{F}_i(t) \quad (38)$$

$$\vec{v}_i(t + \Delta t) = \vec{v}_i(t) + \frac{\Delta t}{2m_i} \left(\vec{F}_i(t) + \vec{F}_i(t + \Delta t) \right) \quad (39)$$

Finally, there are different methods to fix the temperature of the system, which is defined as:

$$T = \frac{2\langle K \rangle}{N_{df} k_B} \quad (40)$$

where $\langle K \rangle$ is the averaged kinetic energy, N_{df} is the number of degrees of freedom accessible to the system and k_B is the Boltzmann constant.

This fixation can be reached scaling the velocities:

$$\vec{v}_{i,scaled}(t) = \vec{v}_i(t) \sqrt{\frac{T_{ref}}{T_t}} \quad (41)$$

- T_t : the instantaneous temperature of the system
- T_{ref} : the reference temperature of the system.

or using an external bath to calculate the velocities. This algorithm was developed by Berendsen et al.^[124] and modifies the equation of motion in the following way:

$$\vec{a}_i(t) = m_i \vec{F}_i(t) + \frac{1}{2\tau_i} \vec{v}_i(t) \left(\frac{T_{bath}}{T_t} - 1 \right) \quad (42)$$

- T_{bath} : the temperature of the external bath
- τ_i : the coupling constant which determines the strength of the coupling to the external bath.

This added term works as a frictional force, i.e., when T_t is higher than the value that we need, the force is negative, so the particles are slowed down and, therefore, the kinetic energy and temperature are reduced. Whereas, if T_t is lower than the value that we need, the reverse happens. With this approximation, we obtain continuous trajectories.

6. Computational Methods

We should also comment that, in our case, the volume is controlled applying periodic boundary conditions.

In order to find out the precision of our simulations, we can check if our system keeps the Eckart's conditions: ^[111] it conserves the total momentum (it is conserved if there is no net force), the angular momentum (it is conserved if there is no torque force) and the total energy (it is conserved as the Hamiltonian is time-independent).

We should also point out that, to choose the initial velocities of the system, we use a Maxwell-Boltzmann distribution for a particular temperature.

It is also important to fix the conditions of the simulation. The most common case is working in constant volume, temperature and total number of particles, what is known as canonical or NVT ensemble, which is the one used the most by experimentalists.

6.5. Free energy in statistical mechanics

There are different techniques for calculating free energies. It can distinguish three different families of methods:

- i) Methods that sample the system in equilibrium.
- ii) Nonequilibrium sampling techniques.
- iii) Methods that introduce additional degrees of freedom, along which the free energy is calculated.

In the first group, it can be included *Thermodynamic Perturbation* or *Free Energy Perturbation* methods (FEP)^[125-128] and *Thermodynamic Integration* methods (TI).^[129] The FEP method is called *Slow Growth* method, and requires that the increase in the reaction coordinate, λ , is slow enough for the system to remain essentially at equilibrium all times. The difference between FEP and TI is that the former averages over finite differences in energy functions, while the latter averages over a differentiated energy function.

The nonequilibrium techniques involve the one discovered by Jarzynski^[130-136], who demonstrated the equivalence for the free-energy change and an exponential average over the work along nonreversible paths originating from a canonic ensemble. This can be exploited in practical simulations moving a constraint on the reaction coordinate relatively fast from an equilibrated system to the target system^[137,138]. This method becomes known as *Fast Growth*.

In the last group can be included techniques such as *Transition-path sampling* (TPS) which uses Monte Carlo importance sampling in the space of trajectories connecting the reactant with the product basin, yielding an ensemble of reactive trajectories^[139-149]. All that is required are low dimensional order parameters that are able to unambiguously separate reactant from product configurations. Other technique belonging to this group is the *Metadynamics* approach^[150-159] which is capable of exploring the free-energy surface without prior information about the location of minima or transition states. Other technique is *Phase-space sampling*, it can be enhanced in constant temperature MD by coupling selected degrees of freedom to a separate thermostat and keeping them at higher temperature than the remainder of the system. Such techniques are known as *adiabatic free-energy dynamics*^[160,161] or *canonical adiabatic free-energy sampling*^[162]. The last one belonging to this group is the one developed by Lu and Yang^[163]. They extended the concept of the reaction-path Hamiltonian^[164] to large QM/MM systems. Using the energies, vibrational frequencies and electronic response properties of the QM region along QM/MM reaction path, they construct a harmonic reaction path potential.

6.5. Free energy in statistical mechanics

Then the FEP technique is presented because is the one it is used in this PhD Thesis.

Using the canonical ensemble, the observable obtained for the free energy is the Helmholtz potential, A , which measures the “useful” work obtained from a system isolated from its surrounding environment at a constant temperature, and it is defined as:

$$A = -k_B T \ln Q_{NVT} \quad (43)$$

Q_{NVT} is the partition function for a system formed by N indistinguishable particles at a constant volume and temperature, i.e., it is the probability to find the system in a certain state:

$$Q_{NVT} = \frac{1}{h^{3N} N!} \int e^{-\left(\hat{H}(\vec{p}, \vec{r}) / k_B T\right)} d\vec{r} d\vec{p} \quad (44)$$

where Q_{NVT} is a property that depends on the energy of each configuration:

$$Q_{NVT} \propto \left\langle e^{-V/k_B T} \right\rangle \quad (45)$$

From this equation, it might be thought that if we evaluate the average on equation (44) along a MD trajectory, we will straightforward obtain the free energy. But this is not the case, because it is very difficult to obtain reliable averages due to the fact that simulations will preferentially explore the configurations with a lower potential energy because they have higher probabilities. To avoid this problem, we resort to the *Umbrella Sampling* technique^[165] (see Figure 20), which allows to explore high-energy zones that would not be explored if the system was left to evolve freely.

Figure 20. Representation of the Umbrella Sampling technique, where the energy profile is divided into several windows.

6. Computational Methods

We compute a particular kind of free energy, the Potential of Mean Force (PMF), which has the same equation as the Helmholtz potential but the averages are made on all the degrees of freedom of the system except along one that corresponds to the distinguished coordinate, ξ . It is defined as:

$$U(\xi_0) = c - k_B T \ln \left(\int d\vec{r} \delta(\xi(\vec{r}) - \xi_0) e^{v(\vec{r})/k_B T} \right) \quad (46)$$

- ξ_0 : the value of the degree of freedom ξ for which the PMF is computed
- $\xi(\vec{r})$: the function that relates ξ to the atomic coordinates $\vec{r}_i$
- c : an arbitrary constant which includes all that is independent of ξ

If the average of the distribution function of the coordinate is expressed as:

$$\langle \rho(\xi_0) \rangle = \frac{\int d\vec{r} \delta(\xi(\vec{r}) - \xi_0) e^{v(\vec{r})/k_B T}}{\int d\vec{r} e^{-v(\vec{r})/k_B T}} \quad (47)$$

then, the PMF is:

$$U(\xi_0) = c' - k_B T \ln \langle \rho(\xi_0) \rangle \quad (48)$$

where c' is another arbitrary constant.

To perform a correct computation of the whole density along ξ_0 (to obtain each value of $\langle \rho(\xi_0) \rangle$), we should make a series of calculations for sampling in different regions of the space (or windows), but this regions should overlap in order to cover the whole configurational space and make the integration possible. Since, as stated before, it would be easier for the system to access to low-energy zones, we need to use an umbrella potential that can focus the sampling for a given value of ξ_0 . Then, the trajectories of each window are added to obtain the full distribution function valid for the whole range of the coordinate ξ_0 . Usually, a harmonic potential is chosen as an umbrella potential:

$$v_{umb} = \frac{1}{2} k_{umb} (\xi_0 - \xi_{ref})^2 \quad (49)$$

where ξ_{ref} is the reference value of the reaction coordinate which is changed at each window; and k_{umb} is the force constant for the potential. Finally, using the *Weighted Histogram Analysis* (WHAM),^[166] we can reconstruct the complete distribution functions computed separately for each window,

6.5. Free energy in statistical mechanics

obtaining the averaged free energy of the system, which, eventually, can be compared to the experimental data (Figure 21).

Figure 21. Representation of the WHAM method. The complete distribution function is obtained from the distribution functions of each window.

6.6. Transition State Theory

Thanks to Statistical Thermodynamics, it is possible to relate the microscopic properties with the macroscopic properties.

In 1938 Wigner^[167] succinctly summarized four conditions required for transition state Theory to be exact. As pointed out by D. G. Truhlar^[168]:

- (1) The reactant region of phase space is populated according to a Maxwell-Boltzmann distribution.
- (2) The Born-Oppenheimer separation of electronic and nuclear motion is valid, so only one potential energy surface is involved (Processes in which electronic motion follows nuclear motion without an electronic state change are called electronically adiabatic, which strictly means that the electronic quantum numbers are conserved)
- (3) The nuclear motion is classical.
- (4) All trajectories crossing the transition state (which is a phase-space hypersurface dividing reactants from products) toward products must have originated on the reactant side and must cross the hypersurface only once.

Condition (4) is now usually called the “no recrossing” assumption. If the reaction coordinate were indeed globally separable, there would be no recrossing. Thus condition (4) is sometimes replaced by the assumption that reaction coordinate is separable. However the reaction coordinate is never globally separable and is at best locally separable, whereas the no-recrossing condition may still be satisfied.^[169]

In this chemical reaction:

where $A^\ddagger$ is the transition state of the (3) postulate, $K^\ddagger$ is the equilibrium constant between A and $A^\ddagger$, and k is the first-order rate constant. According to the third postulate, the reaction rate is:

$$v_{\text{reaction}} = v[A^\ddagger] = \frac{k_B T}{h} [A^\ddagger] \quad (48)$$

Taking into account the definition of the equilibrium constant, $K^\ddagger$, and its relationship with the Gibbs’ free energy:

6.6. Transition State Theory

$$K^\ddagger = \frac{[A^\ddagger]}{[A]} \quad (49)$$

$$\Delta G^\ddagger = -RT \ln K^\ddagger \quad (50)$$

we can obtain the concentration of the species in the transition state:

$$[A^\ddagger] = [A] e^{\left(\frac{-\Delta G^\ddagger}{RT}\right)} \quad (51)$$

and, comparing equation (48) to equation (51):

$$v_{reaction} = \frac{k_B T}{h} [A] e^{\left(\frac{-\Delta G^\ddagger}{RT}\right)} = k[A] \quad (52)$$

we obtain the rate constant for our reaction, the equation:

$$k = \frac{k_B T}{h} e^{\left(\frac{-\Delta G^\ddagger}{RT}\right)} \quad (53)$$

According to equation (53), we could estimate the reaction rate if we are able to determine the free energy between reactants and the transition state, $\Delta G^\ddagger$. (See Figure 4)

7. Publications

**7.1. Publication 1: “Computational Study
on Hydrolysis of Cefotaxime in Gas Phase
and in Aqueous Solution.”**

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<http://dx.doi.org/10.1002/jcc.23030>

Volume 33 | Issues 23–24 | 2012
Included in this print edition:
Issue 23 (September 5, 2012)
Issue 24 (September 15, 2012)

Journal of COMPUTATIONAL CHEMISTRY

Organic • Inorganic • Physical
Biological • Materials

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Hydrolysis of Cefotaxime in Gas Phase and in Aqueous Solution

A theoretical study of the hydrolysis of a β -lactam antibiotic, cefotaxime (CEF), in gas phase with semi-empirical and density functional theory (DFT) methods, and in aqueous solution by means of hybrid quantum mechanics/molecular mechanics (QM/MM) potentials, is presented on page 1948 by Conchín Meliá, Silvia Ferrer, Vicent Moliner, Iñaki Tuñón, and Juan Bertrán. The inclusion of the full molecule of the CEF in the gas phase molecular model has been demonstrated to be crucial since its carboxylate group can activate the nucleophilic water molecule. In aqueous solution, the free energy profiles show a step-wise mechanism that is kinetically determined by the nucleophilic attack of a water molecule which, in turn, can be activated by a proton transfer from the carboxylate group of the substrate (the first step). The image represents the molecule of CEF and the water molecule that hydrolyzes the β -lactam ring.

Parallelization of Grid-Based Algorithms and Implementation in DelPhi

As presented by Chuan Li, Lin Li, Jie Zhang, and Emil Alexov on page 1960, the image shows the distribution of electrostatic field mapped onto the surface of the adeno-associated virus, PDB ID 3KIC. The structural file, after a standard protonation, contains approximately 484,500 atoms. The corresponding potential map is obtained by numerically solving the Poisson-Boltzmann equation in a box domain of size $407\text{\AA} \times 407\text{\AA} \times 407\text{\AA}$ and $815 \times 815 \times 815$ mesh points in total via the MPI-parallelized DelPhi program utilizing 100 CPUs. The iteration algorithm converged within less than three minutes at 100 CPUs without reaching the saturation point, indicating that employing more CPUs will further reduce the computational time.

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MOVIPAC: Vibrational Spectroscopy with a Robust Meta-program for Massively Parallel Standard and Inverse Calculations

Markus Reiher et al.

DOI: 10.1002/jcc.23036

The molecular structure of a model π -helix together with its Raman Optical Activity spectrum is presented and analyzed.

Prediction of Hydrocarbon Enthalpies of Formation by Various Thermochemical Schemes

Angela K. Wilson et al.

DOI: 10.1002/jcc.23038

The correlation consistent composite approach and the G4, G3, and G3(MP2) methods have been used to compute the enthalpies of formation for 60 closed-shell, neutral hydrocarbon molecules. The accuracies of these composite approaches are compared using atomization, isodesmic, and hypohomodesmotic reactions.

Interfacing the GROMOS (Bio)molecular Simulation Software to Quantum-Chemical Program Packages

Wilfred F. van Gunsteren et al.

DOI: 10.1002/jcc.23047

The quantum-chemical/molecular-mechanical functionality of the GROMOS software for (bio)molecular simulation is described. The effect of truncating the QM/MM electrostatic interactions at various cutoff radii is discussed and the application of semi-empirical versus density-functional Hamiltonians for a solute molecule in aqueous solution is compared.

Computational Study on Hydrolysis of Cefotaxime in Gas Phase and in Aqueous Solution

Conchín Meliá,^[a] Silvia Ferrer,^[a] Vicent Moliner,^{*,[a]} Iñaki Tuñón,^{*,[b]} and Juan Bertrán^[c]

We are presenting a theoretical study of the hydrolysis of a β -lactam antibiotic in gas phase and in aqueous solution by means of hybrid quantum mechanics/molecular mechanics potentials. After exploring the potential energy surfaces at semiempirical and density functional theory (DFT) level, potentials of mean force have been computed for the reaction in solution with hybrid PM3/TIP3P calculations and corrections with the B3LYP and M06-2X functionals. Inclusion of the full molecule of the antibiotic, Cefotaxime, in the gas phase molecular model has been demonstrated to be crucial since its carboxylate group can activate a nucleophilic water molecule. Moreover, the flexibility of the substrate implies the existence of a huge number of possible conformers, some of them implying formation of intramolecular hydrogen bond interaction that can determine the energetics of the

conformers defining the different states along the reaction profile. The results show PM3 provides results that are in qualitative agreement with DFT calculations. The free energy profiles show a step-wise mechanism that is kinetically determined by the nucleophilic attack of a water molecule activated by the proton transfer to the carboxylate group of the substrate (the first step). However, since the main role of the β -lactamase would be reducing the free energy barrier of the first step, and keeping in mind the barrier obtained from second intermediate to products, population of this second intermediate could be significant and consequently experimentally detected in β -lactamases, as shown in the literature. © 2012 Wiley Periodicals, Inc.

DOI: 10.1002/jcc.23030

Introduction

β -lactam antibiotics are the most effective chemotherapeutic agents for the treatment of bacterial infections,^[1,2] accounting for more than half of the world's antibiotic market. The mechanism of the antibacterial activity of β -lactams involves the inhibition of the biosynthesis of the bacterial cell wall peptidoglycan. Nevertheless, despite much progress in antibiotics design has been done during the past decades, the increasing use of these compounds has induced the development of different resistance mechanisms in pathogenic microorganisms.^[3] One of the strategies evolved by bacteria to defend themselves from antibiotics is the production of β -lactamases,^[4] which hydrolyze the cyclic four-membered ring to biologically inactivate the drug (Scheme 1).

Today, more than 500 β -lactamases are known, classified into four groups,^[5] A–D, according to their amino acid sequence.^[6] Groups A, C, and D, also called serine- β -lactamases (S β LS), utilize an active site serine as a nucleophile,^[3] while B group requires 1 or 2 Zn(II) ions to perform the hydrolysis and thus are called metallo- β -lactamases (M β LS).^[7] There has been a growing concern about zinc-dependent β -lactamases since, despite catalyzing the same reaction, it seems that S β LS and M β LS do not share any structural nor mechanistic similarity^[8] and, in fact, the latter are unaffected by all clinically useful inhibitors of the serine-active enzymes.^[9] Thus, a detailed knowledge of the hydrolysis of the four-membered ring of β -lactam antibiotics reaction mechanism is required to know the possible ways of inhibiting bacteria activity. It is accepted that hydrolysis includes two steps; nucleophilic attack on the carbonyl group of the β -lactam ring and protonation of N atom

with concomitant scission of the carbon–nitrogen bond. In this regard, there is still a question of debate on the timing of carbon–nitrogen bond scission and the protonation of the N, which could even take place concertedly.

To obtain a theoretical insight into the hydrolysis reaction mechanism, the full structure of known antibiotics together with a water molecule can be used as a truncated molecular model. Many theoretical studies on the β -lactams reactivity have used this strategy, since the early gas phase semiempirical CNDO molecular modelling by Boyd et al.^[10,11] In these papers, the authors studied the attack of a hydroxyl group to a β -lactam ring of a model cephalosporin structure. Obviously, although preliminary studies in gas phase can render important information (such as the required level to describe the chemical process or the selection of the most appropriate

[a] C. Meliá, S. Ferrer, V. Moliner
Departamento de Química Física y Analítica, Universitat Jaume I, 12071
Castellón, Spain
E-mail: moliner@uji.es

[b] I. Tuñón
Departamento de Química Física, Universitat de València, 46100 Burjassot,
Spain
E-mail: ignacio.tunon@uv.es

[c] J. Bertrán
Departament de Química, Universitat Autònoma de Barcelona, 08193
Bellaterra, Spain

Contract/grant sponsor: Ministerio de Ciencia e Innovación; Contract number: CTQ2009-14541; Contract/grant sponsor: Universitat Jaume I—BANCAIXA Foundation; Contract number: P1-1B2011-23; Contract/grant sponsor: Generalitat Valenciana; Contract numbers: Prometeo/2009/053, ACOMP/2012/119, and ACOMP/2012/243.

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Scheme 1. Hydrolysis of the four-membered ring of β -lactam antibiotics.

distinguished reaction coordinate), the results can not be used as the reference reaction to be compared with an enzyme catalyzed reaction, as pointed out by Warshel and coworkers^[12] in a related study on the general base-catalyzed methanolysis of formamide. Massova and Kollman^[13] studied the nonenzymic hydrolysis of β -lactams in gas phase and in solution by means of Hartree-Fock *ab initio* calculations. Frau et al.^[14,15] incorporated solvent effects by supermolecule approaches with a reduced number of water molecules and polarizable continuum dielectric models with several semiempirical and Hartree-Fock Hamiltonians. The molecular mechanism obtained by Frau et al.^[14,15] involved the nucleophilic attack of a hydroxyl ion on the β -lactam carbonyl ring followed by the opening of the ring and, subsequently, the proton of the hydroxyl is transferred to the β -lactam nitrogen. Neutral and alkaline hydrolysis of model β -lactam antibiotics were explored at Hartree-Fock and considering the correlation energy at MP2 level of theory by some of us,^[16] showing that concerted and stepwise mechanism are possible, and the species involved are dependent on the calculation level.

Hybrid quantum mechanics/molecular mechanics (QM/MM) methods have been applied to explore the hydrolysis of acyl-enzyme complex formed by a class A β -lactamase (TEM1) and an antibiotic (penicillanate), as a part of the process of antibiotic's inactivation.^[17] In this study,^[17] where semiempirical Hamiltonians were used to describe the QM region and CHARMM force field to treat protein and solvent molecules, the hydrolysis of the acyl-enzyme was described as a three-step process: the first step corresponding to the proton transfer from a hydrolytic water molecule to the carboxylate group of a glutamate residue and the subsequent formation of a tetrahedral adduct as a consequence of the attack of this activated water molecule to the carbonyl carbon atom of the β -lactam. In the second step, the acyl-enzyme bond was broken, obtaining a negatively charged Ser70 and in the last step this residue was protonated by means of a direct proton transfer from Glu166.

More recently, studies have been focused on M β ls using a water molecule activated by Zn^{2+} cation, thus, generating a hydroxyl ion at biological pH.^[2,7,18–28] A second water molecule, activated by the interaction with the Zn^{2+} cation, has been usually proposed to transfer a proton to the nitrogen atom of the antibiotic ring, and regenerating the hydroxyl group.^[2,19,20,23–26] The hydrolysis of β -lactam ring is usually described by a stepwise mechanism involving an intermediate whose electronic and structural properties depend on the number of Zn cations present in the active site. Thus, while the covalent C–N bond is still formed in the intermediate for

M β ls working with only one Zn cation,^[20,23,24,26] this bond is already broken in the intermediate in M β ls containing two Zn cations.^[19–21] Nevertheless, a concerted mechanism has been also found in the second group of M β ls, with a lower barrier according to calculations carried out including the protein environment.^[20,25,26] These calculations

about M β ls are based on QM/MM molecular dynamics simulations,^[20,24,25] or on supermolecule models at density functional theory (DFT) level within the standard B3LYP hybrid functional.^[18,22,27]

Up to now, many details about β -lactam ring hydrolysis, such as the dependence of the mechanism on the selected theoretical level and on the modelling of the environment effects are still to be solved. We are herein presenting the study of β -lactam ring hydrolysis of Cefotaxime (CEF, see Scheme 2) in gas phase, and in solution by means of hybrid

Scheme 2. Molecule of CEF plus a water molecule.

QM/MM potentials. It is important to point out that gas phase calculations are not performed to examine the hypothesis that enzymes catalyze their reactions by displacing solvent and creating an environment similar to the gas phase for the reacting substrates. This 'desolvation hypothesis' was re-examined by Warshel and coworkers, using an amide hydrolysis as an example,^[29] and showed that enzyme active sites provide specific polar environments that do not resemble the gas phase but that are designed for electrostatic stabilization of ionic transition states and that 'solvate' these states more than water does. Nevertheless, although mechanisms and energy barriers obtained in gas phase calculations can not be used as a model of enzyme catalyzed reactions, as mentioned above, studies in gas phase can render important information of the required level of theory to properly describe a specific kind of chemical transformation, to explore the features of a substrate or to select the proper distinguished reaction coordinate to be used in more complex calculations including the environment effects. Our study, performed at several theoretical levels and methods, will illustrate the basic features of the reaction mechanism and the dependence on the theoretical approach used to afford the analysis of this important chemical problem.

Computational Methods

Potential energy surfaces

The potential energy surfaces (PESs) for the hydrolysis of CEF *in vacuo* have been explored using AM1^[30] and PM3^[31,32] semiempirical methods, typically used in QM/MM potentials, and calculations based on DFT functionals such as B3LYP^[33] and M06-2X.^[34,35] PM3 Hamiltonian has been used to overcome the limitations of AM1 method to describe hydrogen bond interactions, while the highly parametrized M06-2X hybrid functional was selected to improve the limitation of the dispersion treatment of the B3LYP functional. Although other improved functionals have shown to improve the quality of the popular B3LYP functional by including an empirical dispersion correction term, such as B3LYP-D,^[36] recent comparative analysis of DFT functionals support the fact that M06-2X is demonstrated to render slightly more accurate results at slightly lower cost (see for instance Refs.^[37–40]). The standard 6-31G* basis set was used for the DFT optimizations and single point energy calculations have been computed on the obtained structures with the 6-311+G(2df,2p) basis set, that includes diffuse functions. All calculations *in vacuo* were performed with Gaussian 09.^[41]

Effect of aqueous media could be considered pairing DFT methods with PCM implicit solvation model (see for instance Ref. [42]). Nevertheless, we are presenting in this manuscript a study based in a different methodology based in explicit solvation methods, since the future work will be focused in the study of the reaction in the enzyme that, by definition, requires the use of an explicit model to describe the protein environment. In particular, calculations in aqueous solution were performed with the full molecule of CEF and a water molecule described quantum mechanically embedded in cubic box of water molecules (30 Å side, 998 solvent water molecules in total) treated by TIP3P^[43] force field, as implemented in fDYNAMO library.^[44] PM3, B3LYP/6-31G*, and M062X/6-31G* Hamiltonians were used to treat the quantum subsystem. The water molecules were equilibrated by means of 20 ps of hybrid QM/MM MD in reactant state and they were relaxed at every potential energy optimization along the full reaction profile.

Both in gas phase and in solution frequency calculations were performed to verify that the structures represent true minima or first-order saddle point on the gas phase and on the QM/MM PESs. Intrinsic reaction paths were traced down from the corresponding TS structures in both directions to verify that the located TS connects the two minima of the corresponding chemical step. Zero point energies were calculated from the harmonic frequencies. Thermal contributions to the enthalpy and to the free energy were obtained at 298 K by means of the rigid-rotor and harmonic approximation in gas phase.^[45] These approximations were not used to compute free energy contributions in solution, as it has been shown they present important limitations.^[12] No constraints were imposed in any of the optimizations. Natural population analysis has been performed for all stationary structures *in vacuo* and in aqueous solution.^[46]

Potentials of mean force

Potentials of mean force (PMFs) were computed to obtain the free energy reaction profiles in aqueous solution along distinguished geometrical reaction coordinates selected from the results obtained in the PES exploration. It must be point out that in recent years big efforts are focused on obtaining reliable *ab initio*/MM free energies for reactions in the condensed phase at high level. Thus, more advanced approaches that report *ab initio* PMF can be found in the literature such as metadynamics approach^[47] or the recent paradynamics^[48] which is based on the use of an empirical valence bond reference potential, which is similar to the real *ab initio* potential. In this study, we are using a semiempirical Hamiltonian describing the QM regions allows extensive conformational sampling during the dynamics at a reasonable computational cost. As shown below, the main conclusion from this exploration is that the reaction takes place as a combination of three chemical events: (i) the activation of the water molecule by means of a proton transfer to the carboxylate group of CEF (O11 atom, see Scheme 2), and the nucleophilic attack of this activated water molecule to the carbonyl carbon atom (C1), (ii) the scission of the C1–N2 bond, and (iii) the transfer of a proton to the N2 atom. Bidimensional PMFs were then obtained using the C1–O31 and the antisymmetric combination of O31–H32–H32–O11 distances, and the H32–O11 and H32–N2 distances, for the first and third steps, respectively, while a monodimensional PMFs was computed using the C1–N2 distance describing the ring opening, for the second step. Thus, for the first two-dimensional (2D) PMF 15 simulation windows, in a range of distances from 4.28 to 1.48 Å of the C1–O31 coordinate, have been used centered at 20 reference values of the antisymmetric coordinate evenly distributed in a range from –0.79 to 0.86 Å. Sixty simulation windows have been used evenly distributed in a range from 1.59 to 2.79 Å of the C1–N2 interatomic distance to generate the second 1D PMF, and 24 simulation windows in a range of distances from 1.91 to 0.998 Å of the H32–N2 coordinate have been used centered at 24 reference values of the H32–O11 coordinate in a range from 0.98 to 2.54 Å. Umbrella force constant of 2500 kcal mol^{–1} Å^{–2}, were applied to the distinguished reaction coordinates to allow a perfect overlapping among the windows. The weighted histogram analysis method, combined with the umbrella sampling approach,^[49,50] was used to scan the reaction coordinates. 10 ps of relaxation and 20 ps of production, with a time step of 0.5 fs, using the velocity Verlet algorithm^[51] to update the velocities, were run in each window. The PMFs were performed at 300 K, using the NVT ensemble. Structures from the previously obtained QM/MM PESs were used as starting points of each window. A switched cut-off from 10.5 to 14 Å was used for all nonbonded MM interactions.

The activation free energy for a particular reaction can be evaluated from the difference in the value of the 1D PMF between the maximum (the transition state) and minimum (the reactants state).^[52] The activation free energy can be then recovered from the 2D-PMF tracing a maximum probability

Figure 1. Potential Energy Profiles for the hydrolysis of CEF obtained *in vacuo* by means of PM3 (red line), B3LYP/6-31G* (blue line) and M06-2X/6-31G* (green line). Single point energy calculations with B3LYP/6-311+G(2df,2p) (dashed blue line) and with M06-2X/6-311+G(2df,2p) (dashed green line).

reaction path on the 2D-PMF surface and integrating over the perpendicular coordinate, as already presented in our recent studies.^[53,54]

Because of the large number of structures that must be evaluated during free energy calculations, QM/MM simulations were restricted to the use of semiempirical AM1 and PM3 Hamiltonians. As discussed in the following section, the description of the chemical process at the semiempirical level is qualitatively in agreement with DFT/MM calculations, and thus, the free energies obtained using the low level QM description were corrected by means of single point energy calculations at B3LYP and M06-2X level on the stationary point

structures of reactants, intermediates and TSs obtained on the PM3/MM PESs. From the methodological point of view, the comparative analysis of the results obtained on the exploration of the PESs by means of the semiempirical PM3 Hamiltonians and with DFT methods will be of great interest to validate the former for the highly computer demanding hybrid MD simulations, required to get the PMFs.

Results and Discussion

Results *in vacuo*

The potential energy profiles for the hydrolysis of CEF obtained *in vacuo* by means of PM3, B3LYP/6-31G*, and M06-2X/6-31G* levels of theory are presented in Figure 1. Corrections of thermal and zero point vibrational energies, as well as free energy values are reported in Table 1. Although not presented in the figure, the reaction has been also studied with the AM1 semiempirical Hamiltonian, rendering qualitatively the same results as with PM3. Moreover, the effect of the size of the basis set has been also analyzed for the DFT calculations with single point calculations using the 6-311+G(2df,2p) basis set and results are listed in Table 1 and plotted in Figure 1. As observed, the results are in agreement with the ones computed with the standard 6-31G*.

The first conclusion that can be derived from the potential energy profiles is that, while PM3 and M06-2X render a molecular mechanism in three steps (with transition structures TS1, TS2, and TS3), the one obtained with the B3LYP functional takes place in two steps (with transition structures TS1 and TS3). Nevertheless, the potential energy barriers from I1 to TS2

Figure 2. Schematic representation of the structures corresponding to the stationary points located along the B3LYP (a) and M06-2X (b) potential energy profiles for the hydrolysis of CEF in gas phase. Inter-atomic distances (in black) are reported in Å, while charges on key atoms (in red) are in atomic units.

Figure 2. (Continued)

(corresponding to the b-lactam ring opening as mentioned before) located at PM3 and M06-2X levels are very small (1.2 and 1.3 kcal mol⁻¹, respectively). Accordingly, when introducing the zero point vibrational energy (ZPE) corrections, and when computing the enthalpy and free energies, I1 intermedi-

ate disappears in the PM3 profile. In the M06-2X profile, the barrier from I1 to TS2 is almost negligible in terms of enthalpies, 0.3 kcal mol⁻¹, and free energies, 0.1 kcal mol⁻¹. Regarding the third step (corresponding to the protonation of the N2 atom), while the I2 intermediate is a local minimum in the

Figure 3. Structures of reactants and products of the hydrolysis of CEF obtained in vacuo at B3LYP (top) and M06-2X (bottom) level. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1. Relative energies (ΔE) of stationary point structures obtained along the potential energy profiles obtained with PM3, B3LYP/6-31G*, and M06-2X/6-31G* methods in gas phase and from single point energy calculations performed at B3LYP/6-311+G(2df,2p) and M06-2X/6-311+G(2df,2p) levels.

	R	TS1	I1	TS2	I2	TS3	P
PM3							
ΔE	0.0	23.8	-9.1	-7.9	-24.3	2.8	-15.8
$\Delta E + \text{ZPE}$	0.0	22.7	-7.2	-7.4	-22.5	-0.8	-15.0
ΔH	0.0	21.0	-8.5	-8.5	-23.5	-1.8	-16.0
ΔG	0.0	27.2	-3.1	-4.2	-17.5	3.7	-10.6
B3LYP							
ΔE	0.0	14.9	-	-	-8.9	-6.6	-14.6
$\Delta E + \text{ZPE}$	0.0	12.9	-	-	-8.0	-8.0	-12.9
ΔH	0.0	11.8	-	-	-8.7	-8.9	-13.7
ΔG	0.0	14.5	-	-	-6.2	-5.9	-11.0
M06-2X							
ΔE	0.0	12.2	-2.2	-0.9	-10.3	-8.5	-32.6
$\Delta E + \text{ZPE}$	0.0	11.1	-0.5	-0.2	-9.4	-9.9	-30.5
ΔH	0.0	9.9	-1.8	-1.5	-10.0	-10.6	-31.6
ΔG	0.0	13.3	1.9	2.0	-9.5	-9.7	-28.5
B3LYP	0.0	19.1	-	-	-4.6	-2.5	-10.9
M06-2X	0.0	16.8	3.4	4.2	-6.2	-4.5	-27.7

ZPE corrections, relative enthalpies (ΔH), and free energies (ΔG) obtained at 298 K. All values are reported in kcal mol⁻¹.

PM3 and B3LYP free energy profiles, the free energy barrier from this local minimum to the TS3 computed with the later is just 0.3 kcal mol⁻¹. The effect of including these corrections at M06-2X level is more dramatic, since this minimum disappears on the free energy surface, and then the reaction proceeds directly from TS2 to P. As a first important conclusion, the TS1 (corresponding to the activation of the water molecule and the subsequent nucleophilic attack on C1) is the only remaining transition state after inclusion of free energy corrections at all tested theoretical levels. The free energy change from R to TS1, which is then the rate-determining step, ranges from 13.3 to 27.2 kcal mol⁻¹ at the different levels.

Figure 2 shows a schematic representation of the stationary point structures of the B3LYP and M06-2X PESs. The corresponding PM3 and AM1 schematic representation of the stationary point structures are provided as Supporting Information. From the chemical point of view, the first step is described as an attack of the oxygen water molecule to the C1 atom of CEF, concertedly with a proton transfer from the water molecule to the O11 atom of the CEF carboxylate group (Scheme 2). According to B3LYP results, this step implies also the C1–N2 bond breaking, then connecting reactants and I2 through TS1. At the PM3 and M06-2X levels, the C1–N2 bond is not completely broken in the first step, C1 atom presenting sp³ tetrahedral hybridization, and observing an increase of the negative charge on both, O9 and N2 atoms of CEF from reactants to I1. Interestingly, an I1-like intermediate has been previously described in M/ILs containing only one Zn²⁺ cation in the active site.^[20,23,24,26]

In the second step of the M06-2X reaction profile, the C1–N2 bond breaks, which is associated with formation of a sp² planar C1 atom and recovering the double-bond character of the C1–O9 bond in I2. Consequently, the negative charge

on O9 is reduced, in absolute value, from -0.91 to -0.66 a.u., while charge on N2 atom is increased from -0.63 a.u. on I1 to -0.74 a.u. in I2 (Fig. 2). Similar structures to this I2 intermediate have been invoked in mechanisms described in M/ILs containing two Zn²⁺ ions in the active site.^[19–21] From the electronic and geometrical point of view, this intermediate is very similar to the one directly obtained from R at B3LYP level. The large negative charge developed on N2 (-0.74 a.u. obtained with both methods) provokes the hydrogen bond formation with the proton on O11, facilitating the proton transfer to this center. This last step is also different depending on the method. Thus, while B3LYP describes a single proton transfer from O11 to N2, M06-2X describes a very asynchronous concerted double proton transfer; once a proton is almost completely transferred from O11 to N2, the proton initially on O31 is transferred to O11. This double proton transfer is equivalent to a single proton transfer from O31 to N1, assisted by the carboxylic group of CEF, similarly to the mechanism proposed by Olsen et al.^[18,22] where a water molecule or other aminoacids of the active site were suggested to assist the proton transfer. Thus, the resulting product structures are differently protonated forms, being the one obtained by M06-2X about 20 kcal mol⁻¹ more stable than the one obtained with B3LYP (Figs. 1 and 2). The origin of this energetic difference is mainly due to the formation of a strong intramolecular hydrogen bond between O11 and O9 atoms (2.53 Å) at M06-2X level.

To rationalize the differences between the product structures obtained from B3LYP and M06-2X reaction profiles, shown in Figure 3, we have used the B3LYP structure as starting point to carry out a geometry optimization at the M06-2X level and *vice versa*, the M06-2X product structure was used as starting point in a geometry optimization at the B3LYP level. Surprisingly, the new product structure located at the B3LYP level was 10.8 kcal mol⁻¹ more stable than the one obtained from the reaction mechanism, while the new M06-2X structure was 17.1 kcal mol⁻¹ less stable than the presented in its energy profile. Thus, both theoretical levels are able to describe the same product isomers with the same energy ordering but the exploration of the reaction mechanism leads to different final energy minima. The B3LYP product obtained from the PES exploration is not the most stable isomer at this level, while at the M06-2X level the product found corresponds to the most stable isomer form. This different behavior is due to the evolution of the CEF structure along the reaction profiles obtained with the two methods, and it is related to geometrical coordinates that are not directly involved (and controlled) in the reaction progress. This can be easily understood if we compare the reactant structures obtained at the M06-2X and B3LYP levels, provided in Figure 3, which are the starting points in our search on the PES. Although CEF structure is a 'close' conformer at M06-2X, the reactants structure obtained at B3LYP is clearly an 'open' conformer. As previously done for the analysis of products, we have used the B3LYP reactants structure as starting point to perform a geometry optimization at the M06-2X level and *vice versa*. The result is that the 'open' conformation is more stable than the 'close' conformation (by 1.9 kcal mol⁻¹) at B3LYP level, while the 'close'

conformation is the most stable one at M06-2X level (by 4.7 kcal mol⁻¹). This different behavior is probably due to the dispersion contribution included in the M06-2X functional. Consequently, the reactant structures selected to study the reaction profiles were the appropriate ones for each theoretical level.

The evolution of the substrate conformation along the reaction profile can be monitored using several dihedral angles. For instance, the value of N18–C19–C20–C21 dihedral angle in the reactants of B3LYP defines a *trans* conformation (172.1°), while the structure obtained at M06-2X level presents a *cis* conformation (32.2°). The *cis* conformation favors the hydrogen bond interaction between the amino group of N30 and the O16 atom (2.91 Å). This “closed” conformation is maintained along the structures that appear in the M06-2X reaction profile. The structure of products obtained at B3LYP level presents also a “closed” conformation (Fig. 3) that favors the formation of the mentioned hydrogen bond (N30–O16 distance equal to 3.00 Å). However, *cis*–*trans* conformations are not exclusively related with the “open-close” character of the substrate. For instance, N18–C19–C20–C21 dihedral angle is 108.1° and 25.6° in products, at the B3LYP and M06-2X levels, respectively. Another interesting structural feature is the behavior of the dihedral angle defined by atoms of the four-membered ring of CEF: C1–C4–C3–N2. Hence, while in both reactants this angle is close to zero (4.7 and –1.1° in B3LYP and M06-2X, respectively) describing a planar ring, the values obtained in the two products are significantly different (–74.3 and 23.3° in B3LYP and M06-2X, respectively). The C1–N2 breaking bond renders flexibility to the CEF molecular structure allowing the interconversion from “open” to “close” in B3LYP, which is stabilized by the previously mentioned hydrogen bond. These results stress the importance of including the complete structure of CEF in the simulations to get a full picture of the reaction.

The structures of reactants and products located on the semiempirical AM1 and PM3 PESs are similar to the ones obtained at B3LYP level, with open structures on reactants (that do not favor formation of the intramolecular hydrogen bond between the amino group of N30 and the carbonyl O16 atom) and close structures in products (distance between N30 and the O16 equal to 3.00 and 3.32 Å for AM1 and PM3, respectively). In both, AM1 and PM3 PESs, the H31 is not transferred to O11 (see representation of the stationary point structures in Supporting Information). In accordance with previous comment, the “open”–“close” interconversion is observed from I2 on, once the C1–N2 bond is broken.

Hybrid QM/MM results in aqueous solution

Once the hydrolysis of CEF was explored *in vacuo*, the reaction mechanism has been studied in aqueous solution by means of hybrid QM/MM potentials. PESs will be presented first, and once the molecular mechanism was stated, the reaction is explored in terms of free energy profiles.

First of all, the intramolecular N30–O16 hydrogen bond interaction of the CEF molecule observed *in vacuo* does not persist in aqueous solution, where the conformation of the

Figure 4. Potential Energy Profiles for the hydrolysis of CEF in aqueous solution obtained with PM3/MM (light grey line), B3LYP/MM (black lines) and M06-2X/MM (dark grey lines) methods. Solid lines correspond to step-wise mechanism while dotted lines to the concerted one.

substrate is determined by the interactions established with the water molecules, especially due to the electrostatic terms. These solute–solvent intermolecular interactions are favored when the CEF adopts “open” conformations, what is observed at all tested levels of calculations.

The generation of the free energy profiles, in terms of 1D and 2D PMFs, is necessarily limited to the use of semiempirical Hamiltonian to describe the QM region, taking into account the cost of the required expensive QM/MM MD simulations. Thus, a comparative analysis between the semiempirical and DFT PESs is required to validate the results of the following step of our study.

PESs

The resulting hybrid QM/MM potential energy profiles are presented in Figure 4, while schematic representation of the structures corresponding to the stationary points, together with the value of relevant geometrical parameters and atomic charges, are depicted in Figure 5. A list of the relative energies of stationary points is reported in Table 2. The first insight into the profiles reveals that the hydrolysis of CEF in aqueous solution can proceed by two different molecular mechanisms, stepwise and concerted. It is important to point out that both mechanisms have been obtained with the three selected methods. Moreover, the relative energies of all stationary point structures obtained from the PM3/MM and B3LYP/MM profiles can be considered, at qualitative level, as method independent. Regarding the M06-2X/MM method, although I2 is not localized as an intermediate, it does not appear to be kinetically significant according to the PM3/MM and B3LYP/MM profiles.

The reaction has been also studied at AM1/MM level, providing qualitatively the same results as with PM3/MM. A schematic representation of the structures corresponding to the stationary points located along the AM1/MM and PM3/MM potential energy profiles of the hydrolysis of CEF in aqueous solution, together with key interatomic distances are reported

Figure 5. Schematic representation of the structures corresponding to the stationary points located along the hybrid B3LYP/MM (a) and M062X/MM (b) potential energy profiles of the hydrolysis of CEF in aqueous solution. Inter-atomic distances are reported in Å, while charges on key atoms (in brackets) are in atomic units.

Table 2. Relative energies (ΔE) of stationary point structures obtained along the potential energy profiles obtained at PM3/MM, B3LYP/6-31G*/MM and M06-2X/6-31G*/MM level.

	PM3/MM	B3LYP/MM	M06-2X/MM
Concerted mechanism			
R	0.0	0.0	0.0
TSc	44.0	46.4	42.1
P	-26.0	-22.9	-16.5
Stepwise mechanism			
R	0.0	0.0	0.0
TS1	29.6	34.8	27.4
I1	-7.3	12.4	17.4
TS2	-0.1	15.8	21.8
I2	-12.1	3.2	-
TS3	6.9	3.9	-
P	-26.0	-22.9	-16.5

All values are reported in kcal mol⁻¹.

in Supporting Information. A comparison of the potential energy profile obtained at AM1/MM and PM3/MM level is also reported in Supporting Information. As observed in Figure 4, the rate limiting step of the stepwise mechanism, the first step, presents lower barrier than the concerted mechanism, and thus, it must be the relevant pathway from a kinetic point of view. We will then focus on this mechanism.

By comparison with the potential energy profiles obtained *in vacuo*, it seems that aqueous environment increase the barrier associated to TS1 by about 6, 20, and 15 kcal mol⁻¹ at PM3, B3LYP, and M06-2X level, respectively. The origin of this increase of the potential energy barrier comes from the preferential stabilization of reactants rather than by a destabilization of TS1. Analysis of the different energy terms shows that the QM-MM interaction is much more favorable in reactants than in TS1. In particular, the difference of this interaction term between reactants and TS1 is -4, -22, and -17 kcal mol⁻¹ at PM3, B3LYP, and M06-2X level, respectively. This is due to the delocalization of the negative charge in TS1. As deduced from the atomic charges given in Figure 5, the charge on the carboxylate group diminishes from -0.83 a.u. in reactants to -0.72 in TS1 at B3LYP level. Charges variations from -0.81 to -0.71 a.u. have been observed from reactants to TS1 at M06-

2X level. Another important difference between the *in vacuo* and the QM/MM profiles is found in the B3LYP reaction profiles. The I1 intermediate, that was not a stationary point in the *in vacuo* B3LYP PES, appears now as a local minimum stabilized by the aqueous environment. This stabilization is due to the large negative charge (-0.91 a.u.) developed on the O9 atom that can now establish strong hydrogen bonds with solvent molecules. A similar effect has been observed in some enzyme systems by means of the well-known oxyanion hole such as serine proteases,^[55-57] human acetylcholinesterase^[58], ketosteroid isomerase,^[59] and so forth. In general, the geometrical parameters presented in Figure 5 are similar to those found *in vacuo* (Fig. 2) while the charges are larger in aqueous environment due to the polarization effects.

The conclusion that can be obtained from the QM/MM potential energy profiles of the hydrolysis of CEF in aqueous solution is that the reaction preferentially proceeds by means of three chemical steps. The information derived from the QM/MM PESs will be used to select the distinguished reaction coordinate required to study these three steps in terms of PMFs and, considering the qualitative agreement between the PM3/MM and the higher level B3LYP/MM and M06-2X/MM results, the former can be considered as adequate to run the expensive QM/MM MD simulations.

Free energy profiles

The resulting PMFs corresponding to the three steps of the full molecular mechanism of CEF hydrolysis are presented in Figure 6. As mentioned in 'Computational Methods' section, the first step was studied by means of a 2D-PMF using the C1-O31 and the antisymmetric combination of O31-H32-H32-O11 distances (Fig. 6a), a monodimensional PMF for the second step was computed using the C1-N2 distances describing the ring opening (Fig. 6b), and a 2D-PMF using the H32-O11 and H32-N2 distances was generated to study the third step (Fig. 6c).

According to Figure 6a, the first step is a concerted but quite asynchronous mechanism. First, the water oxygen O31 attacks to the C1 atom of CEF, and then, the H32 atom is

Figure 6. Free energy profiles for the three steps of the hydrolysis of CEF in aqueous solution obtained at PM3/MM level: (a) nucleophilic attack of activated water molecule, (b) ring opening and (c) hydrogen transfer to N2.

Table 3. Relative free energies (APMF), with ZPE corrections, of stationary point structures obtained at PM3/MM level in aqueous solution at 298 K.

	R	TS1	I1	TS2	I2	TS3	P
PM3/MM	0.0	42.5	-0.3	1.3	-7.4	24.9	-26.0
+ZPE	0.0	39.9	1.0	1.6	-7.0	20.0	-25.6
B3LYP/MM	0.0	35.1	9.5	2.4	-5.7	4.4	-35.3
+ZPE	0.0	32.5	10.8	2.7	-5.3	-0.5	-34.9
M06-2X/MM	0.0	30.8	4.6	-0.9	-8.3	2.9	-37.4
+ZPE	0.0	28.2	5.9	-0.6	-7.9	-2.0	-37.0

Energy corrections have been done at B3LYP/6-31G*/MM and M06-2X/6-31G*/MM level. All values are reported in kcal mol⁻¹.

transferred to the carboxylate oxygen atom O11. The average values of the key distances defining the position of the TS1 on the 2D-PMF are 1.47, 1.32, and 1.48 Å for the C1—O31, O31—H32, and H32—O11, respectively. Regarding the second step, Figure 6b, the TS2 of the C1—N2 breaking bond can be localized in an early stage of the process, closer to reactants (I1) than to products (I2). The average value corresponding to this intermolecular distance in I1, TS2, and I2 are 1.62, 1.86, and 2.75 Å, respectively. The free energy surface of the third step, Figure 6c, describes a concerted mechanism where the average distances between hydrogen H32 atom and donor (O11) and acceptor (N2) atoms are very close: 1.41 and 1.33 Å, respectively.

To get reliable values of free energy barriers, the PM3/MM results have been corrected at DFT/MM level with the B3LYP and M06-2X functionals as explained in "Computational Methods" section. It must be mentioned that this is not equivalent to generate new PMFs at DFT/MM level, but this would require an extraordinary increase in the computational requirements that is nowadays out of our capabilities. Relative free energies to reactants, computed at the three levels of theory and with ZPE corrections, are reported on Table 3. Thus, the rate limiting step corresponds to the nucleophilic attack of the water molecule and the simultaneous hydrogen transfer to the carboxylate O11 atom of CEF, step 1. The resulting free energy barriers are 42.5, 35.1, and 30.8 kcal mol⁻¹ at PM3/MM, B3LYP/MM, and M06-2X/MM levels, respectively. These barriers are reduced, after ZPE corrections, to 39.9, 32.5, and 28.2 kcal mol⁻¹, respectively. These values are significantly smaller than the barrier obtained by Carloni and coworkers^[24] by means of Car-Parrinello QM/MM simulations (48 kcal mol⁻¹). Although no experimental value has been reported for the hydrolysis of CEF in aqueous solution, our estimation at M06-2X/MM level is close to the free energy barrier for the alkaline hydrolysis of β -lactam compounds related to penicillin at 30.0°C measured by Bowden and Bromley, whose values ranged between 21.1 and 25.4 kcal mol⁻¹.^[60] Nevertheless, CEF hydrolysis was not studied and according to these authors, β -lactam compounds appear to react with rate-determining addition of hydroxide anion but with an increased reactivity arising from the direct action of the fused ring structure and the acylamino side-chain. As a consequence, although a quantitative comparison between theoretical predictions and experimental data is

not possible, our free energy barrier for CEF hydrolysis looks quite reasonable, keeping in mind that the results of Bowden and Bromley obtained for catalysed hydrolysis of different β -lactam compounds in alkaline medium would represent a lower limit.

The second step is described as an exothermic reaction with a very small free energy barrier of 0.5 kcal mol⁻¹ at PM3/MM level after ZPE corrections, in agreement with the Hammond postulate. This I1 intermediate is thus expected to have an extremely short lifetime and, in fact, the barrier from I1 to I2 disappears once the DFT corrections are included. The free energy barrier height of last step appears to be very dependent on the level of theory. Thus, while PM3/MM renders a free energy barrier of 24.4 kcal mol⁻¹, the final values at B3LYP or M06-2X levels, are 2.2 and 3.3 kcal mol⁻¹, respectively. Anyway, this barrier is much lower than the one corresponding to the first step and, consequently, I2 intermediate is not expected to accumulate in aqueous solution.

The full free energy profile is in agreement with previous results. Thus, similar intermediates to I1 and I2 have been described in β -lactamases containing one or two zinc cations in the active site, respectively. Regarding the energetics, it is expected that the catalytic effect of enzymes would dramatically decrease the first and rate limiting step of CEF hydrolysis in aqueous solution. The nucleophile species in the active site of the enzyme is an activated water molecule that after deprotonation produces an hydroxyl anion, stabilized by interactions with one or two Zn atoms. Then, the third step of our mechanism could become rate limiting and second intermediate, I2, could be accumulate in the enzyme, as measured by Benkovic and coworkers^[61,62] in their kinetic studies on Nitrocefin hydrolysis by metallo- β -lactamase of *B. Fragilis*.

Conclusions

Hydrolysis of a β -lactam antibiotic, CEF has been studied *in vacuo* including the full molecule and a water molecule as a nucleophilic agent, and in aqueous solution by means of hybrid QM/MM potentials. First stage of our study has been the exploration of the PESs corresponding to the possible steps *in vacuo* and in solution simulated by a box of explicit water molecules. Inclusion of the full molecule of the CEF in the gas phase molecular model has been demonstrated to be crucial since its carboxylate group can activate the nucleophilic water molecule. Moreover, the flexibility of the substrate implies the existence of a huge number of possible conformers, even in gas phase, some of them implying formation of intramolecular hydrogen bond interaction that can determine the energetics of the conformers defining the different states along the reaction profile. The corresponding PESs have allowed determining the most appropriate distinguished reaction coordinates of every step of the full process, as well as their potential energy barriers. From a computational point of view, the results show that PM3 semiempirical Hamiltonian provides results that are in qualitative agreement with higher level calculations based on DFT; B3LYP and M06-2X hybrid functionals used with standard 6-31G* basis set. This

information has been used to generate the corresponding free energy profiles of the CEF hydrolysis in aqueous solution, in terms of monodimensional and bidimensional PMFs. These profiles, computed at PM3/MM level and corrected with the B3LYP and M06-2X functionals, show a step-wise mechanism which is kinetically determined by the nucleophilic attack of a water molecule activated by the proton transfer to the carboxylate group of the substrate (the first step). Although no experimental value has been found for the hydrolysis of CEF in aqueous solution, our estimation at M06-2X/MM level is meaningfully close to the free energy barrier for the alkaline hydrolysis of β -lactam compounds related to penicillin at 30.0°C measured by Bowden and Bromley.^[60] The small free energy barrier from the first to the second intermediate suggests that accumulation of the former would be insignificant. However, since the main role of the β -lactamase would be reducing the free energy barrier of the first step, the nucleophilic attack to the four-membered ring of the antibiotics, I2 intermediate could be experimentally detected, as measured by Benkovic and coworkers.^[61,62]

Acknowledgments

The authors also acknowledge the Servei d'Informàtica, Universitat Jaume I for generous allotment of computer time.

Keywords: metallo- β -lactamases • M β LS • cefotaxime • reaction mechanism • hydrolysis

How to cite this article: C. Melià, S. Ferrer, V. Moliner, I. Tuñón, J. Bertrán, *J. Comput. Chem.* **2012**, 33, 1948–1959. DOI: 10.1002/jcc.23030

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Received: 26 December 2011
Revised: 10 April 2012
Accepted: 13 April 2012
Published online on 7 June 2012

Supplementary Material

A Computational Study on Hydrolysis of Cefotaxime (CEF) in gas phase and in aqueous solution

C. Melia, S. Ferrer, V. Moliner*

Departamento de Química Física y Analítica; Universitat Jaume I, 12071 Castellón
(Spain)

I. Tuñón*

Departamento de Química Física, Universitat de València, 46100 Burjasot, (Spain)

J. Bertrán

Departament de Química; Universitat Autònoma de Barcelona, 08193 Bellaterra,
(Spain)

Figure S1. Schematic representation of the structures corresponding to the stationary points located along the AM1 (top) and PM3 (bottom) potential energy profiles for the hydrolysis of CEF in gas phase. Inter-atomic distances (in black) are reported in Å.

Figure S2. Structures of reactants and products of the hydrolysis of CEF obtained *in vacuo* at AM1 (top) and PM3 (bottom) level.

Figure S3. Schematic representation of the structures corresponding to the stationary points located along the AM1/MM and PM3/MM potential energy profiles of the hydrolysis of CEF in aqueous solution. Inter-atomic distances (in black) are reported in Å.

Figure S4. Potential energy profiles of the hydrolysis of CEF in aqueous solution obtained at AM1/MM (blue line) and PM3/MM (red line) levels.

7.2. Publication 2: “Theoretical studies of the hydrolysis of antibiotics catalyzed by a metallo- β -lactamase.”

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<https://doi.org/10.1016/j.abb.2015.01.013>

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Theoretical studies of the hydrolysis of antibiotics catalyzed by a metallo- β -lactamase

C. Meliá^{a,*}, S. Ferrer^{a,*}, V. Moliner^{a,*}, J. Bertran^b^a Departament de Química Física i Analítica, Universitat Jaume I, 12071 Castellón, Spain^b Departament de Química, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

ARTICLE INFO

Article history:

Received 6 November 2014

and in revised form 13 January 2015

Available online 23 January 2015

Keywords:

Mono zinc MβLs

CphA

IMI

CEF

QM/MM

Enzyme promiscuity

ABSTRACT

In this paper, hybrid QM/MM molecular dynamics (MD) simulations have been performed to explore the mechanisms of hydrolysis of two antibiotics, Imipenem (IMI), an antibiotic belonging to the subgroup of carbapenems, and the Cefotaxime (CEF), a third-generation cephalosporin antibiotic, in the active site of a mono-nuclear β -lactamase, CphA from *Aeromonas hydrophila*. Significant different transition state structures are obtained for the hydrolysis of both antibiotics: while the TS of the CEF is an ionic species with negative charge on nitrogen, the IMI TS presents a tetrahedral-like character with negative charge on oxygen atom of the carbonyl group of the lactam ring. Thus, dramatic conformational changes can take place in the cavity of CphA to accommodate different substrates, which would be the origin of its substrate promiscuity. Since CphA shows only activity against carbapenem antibiotic, this study sheds some light into the origin of the selectivity of the different MβL and, as a consequence, into the discovery of specific and potent MβL inhibitors against a broad spectrum of bacterial pathogens. We have finally probed that a re-parametrization of semiempirical methods should be done to properly describe the behavior of the metal cation in active site, Zn^{2+} , when used in QM/MM calculations.

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Introduction

β -lactam antibiotics are the most effective chemotherapeutic agents for the treatment of bacterial infections, accounting for more than half of the world's antibiotic market [1,2]. The introduction of β -lactam antibiotics into clinical medicine has had a profound impact on our civilization [3]. The mechanism of the antibacterial activity of β -lactams involves the inhibition of the biosynthesis of the bacterial cell wall peptidoglycan. Nevertheless, despite much progress in antibiotics design has been done during the past decades, the increasing use of these compounds has induced the development of different resistance mechanisms in pathogenic microorganisms [1]. One strategy developed by bacteria to resist the action of antibiotics is the expression of β -lactamases [4] that hydrolyze the four-membered ring of β -lactam antibiotics. It is accepted that hydrolysis involves nucleophilic attack on the carbonyl group of the β -lactam ring and protonation of N atom with concomitant scission of the carbon–nitrogen bond. Nevertheless, the timing of carbon–nitrogen scission bond and the protonation of the N, which could even take place concertedly (see Scheme 1), is an open question of debate. A

detailed knowledge of the hydrolysis of the four-membered ring of β -lactam antibiotics reaction mechanism is required in order to know the possible ways of inhibiting bacteria activity. Nevertheless, this is not an easy task due to the plethora of different β -lactamases identified up to now. Today, more than 500 β -lactamases are known, classified into four groups [5]. A–D, according to their amino acid sequence [6]. Groups A, C and D, also called serine- β -lactamases (SβLs)¹, utilize an active site serine as a nucleophile [1], while B group, or metallo- β -lactamases (MβLs), required 1 or 2 Zn(II) ions to perform the hydrolysis.

The MβLs family was defined in 1997 as a new superfamily of metallohydrolases [7]. There has been a growing concern on this zinc-dependent β -lactamases since, despite catalyzing the same reaction, it seems that SβLs and MβLs do not share any structural nor mechanistic similarity [8] and, in fact, the latter are unaffected by all clinically useful inhibitors of the serine-active enzymes [9]. In fact, no MβL inhibitors are available for clinical use [10]. Another feature of MβLs is their capability of hydrolyzing different

* Corresponding authors.

E-mail addresses: sferrer@uji.es (S. Ferrer), moliner@uji.es (V. Moliner).<http://dx.doi.org/10.1016/j.abbi.2015.01.013>

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¹ Abbreviations used: MD, molecular dynamics; IMI, Imipenem; CEF, Cefotaxime; SβLs, serine- β -lactamases; MβLs, metallo- β -lactamases; PBC, periodic boundary conditions; ABNR, Adopted Basis Newton Raphson; RC, reaction coordinates; WHAM, weighted histogram analysis method; HL, high-level, LL, low-level; DFT, density functional theory; MC, Michaelis complex.

Scheme 1. Schematic representation of the possible reaction mechanism of the hydrolysis of the four membered ring of β -lactam antibiotics: concerted (top) and stepwise (bottom). :B represents a base in the active site of the protein but it can also correspond to a base belonging to the substrate.

antibiotics which means a remarkable substrate promiscuity [11]. Further studies are required to elucidate whether they also show catalytic promiscuity.

Three subgroups of MBL have been further identified depending on sequence structure and activity similarities. B1 and B3 subclasses possess a binuclear active site, which requires one or two Zn(II) ions for full activity and are able to hydrolyze carbapenems, penicillins and cephalosporins [10]. B2 subclass, unlike those from subclasses B1 and B3, are fully active with one zinc ion bound and possess a narrow spectrum of activity, hydrolyzing carbapenem substrates almost exclusively [12]. Initially, a reduced number of structures of B2MBL, CphA from *Aeromonas hydrophila* [13], ImiS from *Aeromonas veronii* bv. Sobria [14] and Sfh-I from *Serratia fonticola* [12], have been crystallized, being the CphA the most studied one. In particular, three different structures, two of them in the apo form and the last one corresponding to the N220G mutant in complex with a biapenem (Bia) derivative, were obtained. Nevertheless, there are some concerns related with these structures. As commented by Garau et al., the electron density could not be interpreted as either biapenem or a hydrolyzed biapenem molecule, although it was clear the presence of two fused rings near the zinc ion and both, C2 and C3 carbon atoms of the intermediate exhibiting sp^3 hybridization [13]. Then, it appears that the molecule has lost the double bond established between these two atoms. Consequently, it is difficult to associate this complex to an intermediate or a product of the antibiotic hydrolysis, as suggested by experimental studies of Sharma et al. for the reaction catalyzed by ImiS [14]. The CphA-Bia complex structure has shown how the zinc metal accommodates in the Zn2 site, with a trigonal bipyramidal coordination formed by Asp120, Cys221, His263, the carboxylate oxygen and the N4 atoms of Bia. Based on these

X-ray structures, Garau et al. suggested a mechanism involving a non-metal-binding water nucleophile, activated by His118 (depicted as “:B” in Scheme 1), that would attack the carbonyl carbon of the substrate, leading to cleavage of the C7–N4 bond of the lactam ring (mechanism “2” in Scheme 1). This proposal has been supported by QM/MM theoretical calculations of Xu et al. [15,16] although suggesting that Asp120 would be the base activating the water molecule, instead of His118. In a more recent paper, Wu et al. [17] proposed a complete reaction mechanism for the hydrolysis of biapenem antibiotic catalyzed by CphA, arguing that the CphA-Bia complex determined by Garau et al. would belong to a minor pathway, in contrast to the original suggestion. In this regard, simulations performed by Gatti [18] suggest that the bicyclic derivative of Garau et al. would not be formed inside the enzyme active site. Hydrolyzed biapenem might be released first, cyclization would occur in solution and then the bicyclic compound would bind back to the active site.

Based on QM/MM calculations, an alternative mechanism was proposed by Simona et al. [19,20] where the nucleophilic attack and the proton transfer to the nitrogen atom of the lactam ring would occur in a single concerted step (see concerted mechanism “1” in Scheme 1). According to this proposal, the mechanism requires the activation of a second catalytic water molecule in the active site of the enzyme. This mechanism would be in agreement with experimental studies of Sharma et al. [14] based on proton inventories showing that at least one proton transfer must be involved in the rate limiting step. Nevertheless, the proposal is based on the existence of a conformation of the Michaelis complex in which the substrate binds the zinc metal through a water molecule. This model is not confirmed by the structural studies of Crowder et al. [21] based on enzyme-product complexes, that

suggest a direct contact between the zinc metal and the carboxylate of the substrate. An initial structure presenting this direct contact was used by Xu et al. [15–17] to propose a step-wise mechanism that renders an estimated free energy barrier for the nucleophilic attack of ca. 14 kcal mol⁻¹, [15] a value in very good agreement with the kinetic experiments of Garau et al. [13]. Nevertheless, this comparison requires the hypothesis that such step was the rate limiting step of the enzymatic cycle, apparently in contradiction with the proton inventory experiments of Crowder et al. [21] and with QM/MM computationally exploration of the full mechanism performed by Simona et al. [19]. In particular, the second step related with the proton transfer from Asp120 to nitrogen atom of substrate, would become the rate limiting step, with a total free energy barrier of ca. 24 kcal mol⁻¹.

Similar debate was open on the mechanisms of binuclear B1 and B3 beta-lactamases. Thus, Dal Peraro et al. [22] proposed a mechanism with nucleophilic attack and proton transfer taking place in a concerted manner, while the simulations of Xu et al. [23] suggest that the reaction would be essentially stepwise, with a first rate limiting nucleophilic attack leading to an intermediate where the negative charge developed in the nitrogen leaving group would be stabilized by one of the Zn metal atoms (Zn2). This stable anionic intermediate, experimentally reported by Benkovic and co-workers [24] and by Vila and co-workers [25], implies a non-negligible energy barrier for the following step. Again, the studies of Dal Peraro et al., on B1 metallo beta-lactamases assumed an initial structure with the carboxylate of the substrate interacting with the zinc ions through a water molecule. This assumption could be in contradiction with reported X-ray crystallographic structures of the enzyme complex with the hydrolysis product of an antibiotic carried out by Spencer et al. that suggests a direct substrate-metal interaction also in reactant complex [26].

Interestingly, β -lactamase catalytic activity has been also studied on B1 class with only one zinc metal in the catalytic pocket based on models with the zinc placed in position 1 [22,27,28]. The activity of B1 enzymes in their mono-nuclear form has been measured for the hydrolysis of penicillin G catalyzed by Co(II) substitute B1 metallo- β -lactamase, BcII [29]. According to this study, the metal was observed in both positions, 1 and 2. Furthermore, a biochemical and biophysical characterization of a B3 class of MbL, GOB-18, has also revealed catalytic activity for the mono-nuclear enzyme form with the zinc ion in position 2.

In a previous paper, we carried out a computational study to explore the hydrolysis of one antibiotic, Cefotaxime (CEF), in gas

phase and in aqueous solution by means of QM/MM potentials [30]. PM3 semiempirical methods rendered results in qualitative agreement with DFT calculations with B3LYP and M06-2X hybrid functionals. The free energy profiles in solution showed a step-wise mechanism kinetically determined by the nucleophilic attack of a water molecule activated by the proton transfer to the carboxylate group of the substrate. As depicted in Scheme 1, this would correspond to the first step of any of the two possible stepwise mechanisms where “B”, in Scheme 1, would represent the carboxylate group of the substrate in this case. According to the barrier obtained from the second intermediate to products, population of the second intermediate would be in agreement with experimentally detected anionic intermediates in β -lactamases [24,25]. A concerted mechanism (see Scheme 1), with a water molecule activated by the nitrogen atom of the substrate was also obtained although with a much higher free energy barrier. Keeping in mind the hypothesis that similar molecular mechanisms take place in solution and in the active site of enzymes [31], we are in this paper exploring these two mechanisms in the active site of a mono-nuclear β -lactamase. In particular, we are studying the hydrolysis of Imipenem (IMI), an antibiotic belonging to the subgroup of carbapenems, and the Cefotaxime (CEF), a third-generation cephalosporin antibiotic, in the active site of CphA from *A. hydrophila* (see Scheme 2). Keeping in mind that CphA shows only activity against carbapenem antibiotic, a comparative analysis of the results obtained for both inhibitors will be used to shed some light into the origin of the selectivity of the different MbL.

Computational methods

The initial coordinates were taken from the 1.90 Å resolution X-ray crystal structure of a mutated CphA from *A. hydrophila* complexed with the hydrolyzed biapenem (BMH) antibiotic (PDB entry 1X81) [13]. IMI and CEF were docked on position initially occupied by BMH. Hydrogen atoms were incorporated into the structure to a state complementary to pH 7.5, using RDYDAMO library [32]. Since standard pKa values of ionizable groups can be shifted by local protein environments [33,34], an accurate assignment of the protonation states of all these residues was carried out by recalculating de standard pKa values of the titratable amino acids with the empirical PROPKA [35,36]. Due to the fact that the total charge of the system was not neutral, four Cl⁻ counterions were placed in optimal electrostatic positions around the protein (never closer than 10.5 Å from any atom of the system or 5 Å from another

Scheme 2. Schematic representation of (a) Imipenem, IMI, and (b) Cefotaxime, CEF, β -lactam antibiotics.

counterion, and using a regular grid of 0.5 Å). Then the system was placed in the mass center of a cavity deleted from a prerelaxed orthorhombic box of water molecules ($80 \times 80 \times 100 \text{ Å}^3$). All the water molecules with an oxygen atom lying within 2.8 Å of any heavy atom were removed.

The entire chemical system was then divided into a QM region described by means of PM3 [37,38] semiempirical method (using zinc parameters optimized for metalloenzymes [39]), and a MM region described with the OPLS-AA and TIP3P [40] force fields for the protein and water molecules, respectively. The QM region comprised the substrate, the Zinc atom, the side chains of Asn116, His118, Asp120, His196, Cys221 and His 263, and the water molecules that were required for the hydrolysis. The MM region contains the rest of the system, including counterions and crystallization and solvation water molecules. Then, the full system contains 94 QM atoms in the system with IMI and 104 QM atoms in the system with CEF (see Scheme 3). The link-atom method [41] was used to treat the covalent bonds of amino acids crossing the boundary between the QM and MM regions, between the c_α and c_β atoms, to satisfy the valence of the QM fragments.

All atoms away from a sphere of 30 Å radius centered in the substrate, were kept frozen during all simulations. For this purpose, a Langevin bath with a coupling temperature of 300 K was employed throughout this work, using the canonical thermodynamic ensemble (NVT). A total run of 200 ps for the whole system was made with an integration step size of 1 fs. Periodic boundary conditions (PBC) and a switch force function with a cutoff distance in the range of 14.5–18 Å were used to treat the nonbonding interactions. After setting up the model, the complete system was optimized using the Adopted Basis Newton Raphson (ABNR) method with the backbone of the protein frozen, and then it was equilibrated by 500 ps QM/MM MD simulation. Analysis of the final structures and the time evolution of the root mean square

deviation of the atoms of the backbone (RMSD, reported in Supporting information) suggested that the system was equilibrated. The last structure from this 500 ps MD simulation was fully optimized again to serve as the reaction reactant for the rest of the study. QM/MM Potential Energy Surfaces, PESs, for the four systems have been computed to explore different mechanisms, and stationary point structures characterized by frequency calculations were located. Afterwards, the free energy profiles of the possible reaction paths were obtained in terms of two-dimensional potential of mean force, 2D PMFs, computed as a function of distinguished geometrical reaction coordinates (RC) deduced from the PESs explorations. In particular, the mechanism found was a concerted mechanism, in particular two RC were chosen, the distance of the nucleophilic attack RC1: d(OH2-C) and the antisymmetric combination of the distances involved in the Nitrogen protonation RC2: d(OH2-H2)-d(H2-N).

The weighted histogram analysis method (WHAM), combined with the umbrella sampling approach [42,43], was employed to scan the reaction coordinates. Umbrella force constant of $2800 \text{ kJ mol}^{-1} \text{ Å}^{-2}$, were applied to the distinguished reaction coordinates to allow a perfect overlapping among the windows. 10 ps of relaxation and 20 ps of production, with a time step of 0.5 fs using the velocity Verlet algorithm [44] to update the velocities, were run in each window. The PMFs were performed at 300 K, using the NVT ensemble. Structures from the previously obtained QM/MM PESs were used as starting points of each window.

The activation free energy for a particular reaction can be evaluated from the difference in the value of the one dimensional PMF between the maximum (the transition state) and the minimum (the reactants state). The activation free energy can be then recovered from the 2D-PMF tracing a maximum probability reaction path on the 2D-PMF surface and integrating over the perpendicular coordinate [45].

Scheme 3. QM region in the different calculation with Imipenen (IMI in black) and Cefotaxime (CEF in grey), link atoms are represented by black dots.

Because of the large number of structures that must be evaluated during free energy calculations, QM/MM calculations are usually restricted to the use of semiempirical Hamiltonians. In order to reduce the errors associated to the quantum level of theory employed in our simulations, a new energy function defined in terms of interpolated corrections was used: [46–48]

$$E = E_{\text{PM3/MM}} + S[\Delta E_{\text{LL}}^{\text{HL}}(\zeta_1, \zeta_2)] \quad (1)$$

where S denotes a two-dimensional spline function, and its argument $\Delta E_{\text{LL}}^{\text{HL}}(\zeta_1, \zeta_2)$ is a correction term evaluated from the single-point energy difference between a high-level (HL) and a low-level (LL) calculation of the QM subsystem. PM3 semiempirical Hamiltonian was used as LL method while a density functional theory (DFT) [49] based method was selected for the HL energy calculation. In particular, HL energy calculations were performed by means of the hybrid M06-2X [50] functional using the standard 6-31+G* basis set, which was proved to render good results in our previous study of the hydrolysis of CEF in solution [30]. In particular, single point energy calculations at M06-2X/MM level were performed from the structures generated in the PM3/MM surfaces: 2891 and 1505 structures for the hydrolysis of IMI and CEF antibiotics, respectively. These calculations were carried out using the Gaussian09 program [51], while all QM/MM calculations were performed with fDYNAMO.

Results

The reaction paths corresponding to the concerted and the step-wise mechanism, equivalent to the mechanisms previously explored in aqueous solution in our lab [30], have been explored by QM/MM PESs. The results show that any attempts to get the PESs corresponding to a step-wise mechanism, describing a proton transfer from a water molecule to the oxygen atom of the carboxylate group of the substrate and the nucleophilic attack to the carbonyl carbon of the beta-lactam ring, was unsuccessfully. Obviously, the fact that the carboxylate group is strongly interacting with the Zn ion rules out the possibility of acting as a proton acceptor. The PESs of the concerted mechanism corresponding to the hydrolysis of IMI and CEF are presented in Fig. 1. The antisymmetric combination of $O_{w1}H_{w1}-H_{w1}N_4$ distances and the $O_{w1}-C_7$ distances were employed to generate the PESs.

As observed in Fig. 1, the hydrolysis of both antibiotics catalyzed by CphA takes place via an asynchronous concerted mechanism. Structures selected from the quadratic region of the saddle point were used to localize and refine the corresponding transition state structures, TSs. Both PESs show how the nucleophilic attack and the proton transfer takes place in a concerted synchronous way from reactants to TS while the process from the TS to products is controlled basically by the coordinate associated to the proton transfer. IRC paths were traced down from these TSs forward and backwards to the corresponding reactants and products valleys. Finally, fully optimizations were carried out to reach the minimum energy structures, confirming the located TSs connect the reactants and products. Key distances of structures of the active site in reactants, transition states and products are listed in Table S1 of Supporting information, for hydrolysis of IMI and CEF, respectively.

Once the PESs were explored, the free energy surfaces, obtained in terms of 2D PMFs, were computed and the results are presented in Fig. 2. Averaged values of key inter-atomic distances obtained in reactants, TS and products of hydrolysis of both antibiotics are listed in Table 1, schematic representation of these states are presented in Figs. 3 and 4, and values of relative energies deduced from the QM/MM 2D PMF and from the PESs, both activation and reaction energies, are listed in Table 2. The free energy surfaces presented in Fig. 2 are in a qualitative agreement with the

Fig. 1. PM3/MM PES of hydrolysis of (a) IMI and (b) CEF antibiotics performed in the active site of the CphA from *Aeromonas hydrophila*.

Fig. 2. PM3/MM 2D PMF of hydrolysis of (a) IMI and (b) CEF antibiotics performed in the active site of the CphA from *Aeromonas hydrophila*.

corresponding PESs presented in Fig. 1. The minimum free energy profile describes a mechanism where the nucleophilic attack of the water molecule to C7 atom and the proton transfer to the N atom of the beta-lactam ring take place in a concerted way. In both cases, once the TS is reached the process is controlled basically by the coordinate associated to the proton transfer.

As observed in Fig. 3, the reactants structure of IMI hydrolysis shows how the Zn appears coordinated to His263, to Asp120 (bifurcated coordination), to a water molecule w2, and to the antibiotic through the carbonyl oxygen and the carboxylate group. The carbonyl bond of IMI seems to be polarized by the water molecule w2 and by the Zn atom, thus activating the C7 atom of the lactam ring for the nucleophilic attack. Another role of w2 molecule is to participate in an indirect coordination between Cys221 and the Zn atom. The reactive water molecule, w1, appears to be oriented by the interaction with Asp120 for the nucleophilic attack to C7.

Table 1
Key interatomic distances obtained from the PM3/MM MD simulations performed at reactants, TS and products of the hydrolysis of (a) IMI and (b) CEF antibiotics performed in the active site of the N220G CphA from *Aeromonas hydrophila* obtained at PM3/MM level. All values are reported in Å.

	IMI			CEF		
	R	TS	P	R	TS	P
d(C7, Ow)	4.25 ± 0.24	1.47 ± 0.00	1.33 ± 0.02	3.65 ± 0.34	1.46 ± 0.00	1.35 ± 0.02
d(Ow, Hw)	0.96 ± 0.02	1.31 ± 0.00	2.84 ± 0.23	0.95 ± 0.02	1.12 ± 0.00	3.38 ± 0.47
d(Hw, N4)	4.43 ± 0.29	1.43 ± 0.00	1.00 ± 0.03	4.12 ± 0.51	1.39 ± 0.00	1.00 ± 0.03
d(C7, N4)	1.46 ± 0.03	1.57 ± 0.00	2.82 ± 0.08	1.47 ± 0.03	2.69 ± 0.00	2.73 ± 0.12
d(O, C7)	1.22 ± 0.02	1.29 ± 0.02	1.24 ± 0.02	1.20 ± 0.02	1.20 ± 0.02	1.22 ± 0.02
d(Zn, O)	2.05 ± 0.05	2.03 ± 0.04	2.04 ± 0.05			
d(Zn, O9)	1.97 ± 0.03	1.97 ± 0.03	1.97 ± 0.03	1.94 ± 0.03	1.94 ± 0.03	1.96 ± 0.03
d(Zn, Asp120-O1)	2.06 ± 0.05	2.07 ± 0.05	2.26 ± 0.41	1.95 ± 0.03	1.95 ± 0.03	2.00 ± 0.05
d(Zn, Asp120-O2)	2.07 ± 0.05	2.08 ± 0.05	2.04 ± 0.05			
d(Zn, His263)	2.07 ± 0.04	2.06 ± 0.04	2.07 ± 0.05	2.04 ± 0.04	2.03 ± 0.04	2.07 ± 0.05
d(Zn, Ow2)	2.06 ± 0.05	2.05 ± 0.04	2.05 ± 0.05			
d(O, Hw2)	1.79 ± 0.11	2.40 ± 0.52	2.43 ± 0.61			
d(Asp120, H2w1)	2.33 ± 0.53	1.77 ± 0.06	2.61 ± 0.54			
d(Zn, Cys221)				2.37 ± 0.08	2.34 ± 0.08	2.45 ± 0.12
d(Hw2-O10)				2.72 ± 0.64	2.04 ± 0.24	2.53 ± 0.53

As the reaction proceeds, the w1 molecule approach to the antibiotic and its interaction with Asp120 is lost after reaching the TS. Asp120 interaction with Zn cation does not change during the full process. Nevertheless, while a perfectly symmetric bifurcated interaction is established in reactants and TS of IMI, the Zn ion is preferentially interacting with only one oxygen atom of the Asp120 in products. According to the average values obtained from the structures derived from the 2D PMF, Asp120 would not follow the displacement of the w1 nucleophilic water molecule during the process in IMI hydrolysis. In fact, the shortest distance between oxygen atom of Asp120 and the hydrogen atom of w1 is observed in the TS (1.77 Å) while this distance is significantly larger in products (2.61 Å).

Structures for the hydrolysis of CEF (see Fig. 4) are significantly different. First of all Zn metal presents a tetrahedral coordination, interacting with the carboxylate group of the antibiotic, His263, Cys221 and Asp120. Interestingly, interaction of Asp120 and Zn ion in this system takes place through a single interaction that does not change during the process (from 1.95 to 2.00 Å). The nucleophilic water molecule w1, is interacting with His118 that orients the molecule for the attack, and not with Asp120 as in the IMI hydrolysis. This w1 water molecule is activated by an interaction with the carboxylate group of the substrate through a water molecule w2. Finally, it is also noticeable the interaction between His118 and Asp120 through a conserved water molecule w3. This interaction, as well as the coordination sphere of the Zn atom does not change along the reaction process from reactants to products, as appeared in the IMI hydrolysis reaction.

A detailed analysis of evolution of C7–O distance, as reported in Table 1, shows how the TS in IMI presents a tetrahedral coordination of the C7 carbon atom of the beta lactam ring (1.29 Å), not observed in reactants or products, where the distance is significantly shorter (1.22 and 1.24 Å, respectively), characteristic of a double bond with a planar conformation. The oxygen atom is basically stabilized by an oxyanion hole through interaction with Zn ion (2.03 Å). The interaction of the carbonyl oxygen established in reactants with the w2 water molecule is stronger than in TS and products, as shown by a shorter interatomic distance (1.79 Å in reactants and 2.40 and 2.43 Å in TS and products, respectively). In CEF hydrolysis, no interaction is observed between O and Zn ion, which is related with the almost unchanged value of C7–O distances during the reaction. The value of the obtained C7–O distance in the three states is characteristic of a double bond (1.20, 1.20 and 1.22 Å in R, TS and products, respectively). These differences are related with the differences observed in the N4–C7 distance. A large value of this distance is observed in the TS of the CEF (2.69 Å) while the value obtained in the IMI TS

(1.57 Å) reveals a completely different nature of the TSs. Analysis of the distances describing the position of the transferred proton from the reactive w1 water molecule to N4 atom of the beta-lactam ring shows how the TS of the hydrolysis of IMI is more dissociative than the TS of CEF hydrolysis. Thus, the distances between the transferred proton and the donor and acceptor atoms obtained in IMI (1.31 and 1.43 Å, respectively) are significantly larger than the values obtained in CEF (1.12 Å and 1.39 Å, respectively). These differences of the TS located for the IMI and CEF hydrolysis indicate CphA, depending on the antibiotic, can use different mechanisms.

Population analysis on reactants and TS in both reactions (see Table S2 of Supporting information) confirms the difference nature of the TSs. The results show how the charge in N4 and carbonyl O atom of the beta-lactam ring are increased by –0.31 a.u. and –0.01 a.u. when going from reactants to TS in the CEF. This trend is completely different in IMI; where the charge on N1 atom is virtually unchanged (from –0.007 to +0.007 a.u.). O atom charge changes from –0.286 to –0.568 a.u. in the TS. As summary, negative charge was located in the O atom of the carbonyl group in the TS of the IMI hydrolysis, while in the CEF the charge is mostly in the N4 atom. This electronic analysis confirms the geometrical analysis and reveals two different mechanisms.

The reaction and activation energies obtained in terms of potential energies ($\Delta E^{\ddagger}$ and ΔE_R) and free energies ($\Delta G^{\ddagger}$ and ΔG_R), for the hydrolysis of IMI and CEF antibiotics performed in the active site of the N220G CphA from *A. hydrophila* obtained at PM3/MM level are reported in Table 2. As observed, both potential energy barriers are quite high, being the reaction of the IMI hydrolysis clearly more favorable (45.5 vs. 53.1 kcal mol^{–1}). Nevertheless, both barriers become almost equivalent when computing the free energy barriers at the same level of theory (39.2 and 39.9 kcal mol^{–1}, respectively). Thus, a significant reduction in the barriers is observed after including the entropic contribution, more dramatic in the case of the CEF hydrolysis. These values, when including the correction at M06-2X/MM level, are 32.6 and 35.6 kcal/mol^{–1}. Interestingly, according to the PM3/MM and M06-2X/MM corrected surfaces, reported in Supporting Information, both methods describe similar reaction mechanisms with almost coincident quadratic regions of the TSs.

Discussion

Michaelis complex (MC). First conclusion that can be derived from comparison of averaged structures of MC of the hydrolysis reaction of CEF and IMI, with the experimental X-ray diffraction

Fig. 3. Schematic representation of PM3/MM located structures of reactants, TS and products of the hydrolysis of IMI antibiotics catalyzed by the N220G CphA from *Aeromonas hydrophila*.

structure 1X8I [13] is that both MC structures are quite different (see Fig. 5). As observed, the structure of CEF in the Michalis complex is similar to the holo-enzyme X-ray structure obtained by Garau et al. [13]. The structure of protein complexed with the bis substrate solved by Garau et al., that do not correspond to the reactants; neither an intermediate, is comparable with the structure of MC of CEF obtained after the MD simulations. Also, the experimental results of Garau et al. suggest that the nucleophilic water

molecule would be activated by interaction with the His196 residue, as also indicated by our calculations. On the contrary, the MC obtained for the hydrolysis of IMI presents dramatic differences with respect to the experimental structures of the apo or the protein-substrate complex determined by Garau. In particular, the Asp120 interacts in a bifurcated way with the Zn ion, the carbonyl oxygen of the beta lactam ring interacts directly with the Zn ion thus being activated for a nucleophilic attack, and finally, a

Fig. 4. Schematic representation of PM3/MM located structures of reactants, TS and products of the hydrolysis of CEF antibiotics catalysed by the N220G CphA from *Aeromonas hydrophila*.

direct interaction between the metal and the Cys221 is not observed in the structures after our QM/MM MD simulations (see Fig. S1 of Supporting information). Interestingly, this result has been reproduced when preparing the molecular system with Lys224 in a non-protonated state (as predicted by the PROPKA2) but also when repeating the simulations after preparing the system with the protonated Lys224 (as predicted by PROPKA3). It can be then concluded that lack of coordination of Cys221 with the Zn ion does not depend on the protonation state of Lys224.

Table 2
Reaction and activation energies obtained in terms of potential energies ($\Delta E^\ddagger$ and ΔE_R) and free energies ($\Delta G^\ddagger$ and ΔG_R), for the hydrolysis of IMI and CEF antibiotics performed in the active site of the N220G CphA from *Aeromonas hydrophila* obtained at PM3/MM and after correction at M06-2X/MM level ($\Delta G_{corr}^\ddagger$ and $\Delta G_{R,corr}$). All values in kcal mol⁻¹.

	$\Delta E^\ddagger$	ΔE_R	$\Delta G^\ddagger$	ΔG_R	$\Delta G_{corr}^\ddagger$	$\Delta G_{R,corr}$
IMI	45.5	-9.2	39.2	-6.2	32.6	-9.8
CEF	53.1	-12.9	39.9	-11.2	35.6	-17.5

complex not corresponding to the reactants or intermediate, none of the obtained structures could be, a priori, discarded.

The problem related with the plasticity of metal ions in the active site of proteins has been also discussed in enzymes belonging to the Alkaline Phosphatase superfamily, that contains two Zn ions in the active site. Theoretical simulations performed with hybrid QM/MM methods, including specific d-orbital description in the semiempirical AM1 Hamiltonian, was found that movement of Zn ions is dramatic from R to TS [55–58]. Nevertheless, we must consider the possibility that, as mentioned by Hou and Cui [59], the main cause for the large structural variations in our simulations [55–58] was the use of AM1 to describe the metal ion. The question of whether the large mobility of the metals in this kind of enzymes is an artifact of the computing methods or reveals the plasticity of the metals in the active site requires further validation to provide a definitive answer, as recently proposed in a perspective paper by Kamerlin and co-workers [60]. In particular, there are no experimental X-ray structures of the Michaelis complex of mono-nuclear metallo- β -lactamases with different substrates to confirm the observations obtained in the present paper, where the QM/MM MD simulations have been performed using the semiempirical PM3 hamiltonian. In order to test, at some extent, the parametrization of PM3 for Zn^{2+} metal ion, analysis of charges of CEF in active site of the protein has been performed at PM3 and M06-2X/6-31G*(d,p) level of theory. The results, presented in Fig. 6, show the large difference of charge transfer between the metal and its coordination sphere that, in turn, would suggest an artifact of the PM3 parametrization of the Zn ion. Some of the observations obtained during the QM/MM MD simulations could then be attributed to this possible deficiency.

Conclusions

In this paper, hybrid QM/MM molecular dynamics (MD) simulations have been performed to explore the mechanisms of hydrolysis of two antibiotics, IMI (an antibiotic belonging to the subgroup of carbapenems) and CEF (a third-generation cephalosporin antibiotic), catalyzed by a mono-nuclear β -lactamase, CphA from *A. hydrophila*. The calculations have allowed obtaining, not only the free energy profiles that determine the mechanisms and the energetics of the processes, but also a deep conformational analysis of the active site. Regarding the two explored mechanisms, the hydrolysis of the four-membered ring of β -lactam antibiotics takes place through a concerted mechanism where the nucleophilic attack on the carbonyl group of the β -lactam ring, the protonation of the N atom and scission of the carbon–nitrogen bond take place in a single chemical step. Any attempt to obtain the stepwise mechanism, which is the most favorable reaction path for hydrolysis of these two antibiotics in aqueous solution [30] was unsuccessful. A deeper comparative analysis of the two reactions in CphA reveals electronically different transition state structures: while the TS of the CEF is a ionic species with negative charge on nitrogen, the IMI TS presents a tetrahedral-like character with negative charge on oxygen atom of the carbonyl group of the lactam ring. According to our results, dramatic conformational changes can take place in the cavity of CphA to accommodate different substrates, which would be the origin of its substrate promiscuity. This feature of the β -lactamase would be in turn, associated to the different mechanisms that the protein employs to hydrolyze the different antibiotics: i.e. the catalytic promiscuity.

From the energetic point of view, the PMFs obtained at the highest level of theory used in the present study, when including the correction at M06-2X/MM level, render free energy barriers of 32.6 and 35.6 kcal/mol⁻¹ for the hydrolysis of IMI and CEF, respectively. This trend would be in agreement with the fact that

CphA shows only activity against carbapenem antibiotic. Nevertheless, in our previous study in aqueous solution [30], a substrate assisted mechanism presented a barrier of 30.8 kcal mol⁻¹ at M06-2X/MM level. Then, although possible, the explored mechanism does not seem to be the one taking place in the mono metallo- β -lactamase proteins, considering the obtained free energy barriers in both media. Our results suggest that the protein could have a chemical role in the catalytic process by favoring the reaction to progress through the existence of an intermediate. This is in agreement with complex concerted mechanisms involving participation of an additional water molecule, as proposed by Simona et al. [20], or in stepwise mechanism with participation of Asp120 as proposed by Wu et al. [17], or His196 as suggested by Garau et al. [13]. Our results, together with these previous studies, demonstrate the complexity of the enzyme reaction mechanisms in mono metallo- β -lactamases. Our proposal of the proton transfer from a water molecule to the nitrogen atom of the substrate could be enlarged by exploring other mechanism [52]. In most of the cases, reaction coordinate would involve participation of the environment and further more complex explorations of free energy surfaces would be required. The complete computational study will then be used to shed some light into the origin of the selectivity of the different M β L and, as a consequence, into the discovery of specific, potent M β L inhibitors against a broad spectrum of bacterial pathogens.

Acknowledgments

We thank FEDER and the Spanish Ministerio de Economía y Competitividad (project CTQ2012-36253-C03), Universitat Jaume I (project P1 1B2011-23) and Generalitat Valenciana (PROMETEOII/2014/022 and ACOMP/2014/277 projects). The authors also acknowledge the Servei d'Informàtica of the Universitat Jaume I for generous allotment of computer time.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.abb.2015.01.013>.

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Supporting Information

**Theoretical studies of the hydrolysis of antibiotics catalized by a
Metallo- β -lactamase.**

C. Melia, S. Ferrer, V. Moliner, J. Bertran

Table S1. Key interatomic distances obtained from the stationary point structures located in the PESs

	PES		
IMI	R	TS	P
d(C7,Ow)	3.74	1.46	1.33
d(Ow,Hw)	0.96	1.32	3.51
d(Hw,N4)	4.26	1.43	1.00
d(C7,N4)	1.46	1.60	2.53
d(O,C7)	1.22	1.29	1.25
d(Zn, O)	2.05	2.03	2.04
d(Zn, O9)	1.97	1.98	1.97
d(Zn, Asp120-O1)	2.05	2.06	2.06
d(Zn, Asp120-O2)	2.05	2.05	2.06
d(Zn, His263)	2.07	2.07	2.07
d(Zn, Ow2)	2.05	2.04	2.04
d(O,Hw2)	1.80	1.77	1.80
d(asp120,H2w1)	1.79	1.80	1.79

CEF	R	TS	P
d(C7,Ow)	3.07	1.46	1.34
d(Ow,Hw)	0.95	1.04	2.44
d(Hw,N4)	3.80	1.62	1.00
d(C7,N4)	1.48	2.17	2.76
d(O,C7)	1.20	1.20	1.22
d(Zn, O9)	1.96	1.96	1.95
d(Zn, Asp120)	2.28	2.29	2.28
d(Zn, Cys221)	2.03	2.03	2.03
d(Zn, His263)	1.95	1.95	1.95
d(Hw, Ow2)	2.09	1.98	2.25
d(H2w1, S-His118)	2.89	4.59	4.81
d(Asp120,Hw3)	1.76	1.74	1.77
d(Hw2-O10)	1.91	1.81	1.89

3

Table S2. Population analysis on key atoms computed in reactant complex (R) TS and products (P).

CEF		
	O	N
R	-0.292	-0.051
TS	-0.303	-0.36
P	-0.463	0.058
IMI		
	O	N
R	-0.286	-0.007
TS	-0.568	0.007
P	-0.407	0.035

4

Figure S1. Time evolution of key distances during the QM/MM MD simulations of IMI in active site of CphA from *Aeromonas hydrophila*: O18 – Zn²⁺ (blue line), O17 – Zn²⁺ (green line), SCys221 – Zn²⁺ (red line) and NLYS224 – O17 (purple line).

Figure S2. Time evolution of the RMSD along the QM/MM MD performed for the heavy atoms of the backbone of the CphA from *Aeromonas hydrophila* with IMI (green) and CEF(red) in the active site.

Figure S3. PM3/MM 2D PMF of hydrolysis of (a) IMI and (b) CEF antibiotics performed in the active site of the CphA from *Aeromonas hydrophila* corrected at M06-2X/MM level. See text for details.

7.3. Publication 3: “Investigation of the Hydroxylation Mechanism of Noncoupled Copper Oxygenases by Ab Initio Molecular Dynamics Simulations.”

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<http://dx.doi.org/10.1002/chem.201301000>

Investigation of the Hydroxylation Mechanism of Noncoupled Copper Oxygenases by Ab Initio Molecular Dynamics Simulations

Conchín Meliá,^[a] Silvia Ferrer,^[a] Jan Řezáč,^[b] Olivier Parisel,^[c] Olivia Reinaud,^[d] Vicent Moliner,^{*,[a]} and Aurélien de la Lande^{*,[e]}

Abstract: In Nature, the family of copper monooxygenases comprised of peptidylglycine α -hydroxylating monooxygenase (PHM), dopamine β -monooxygenase (D β M), and tyramine β -monooxygenase (T β M) is known to perform dioxygen-dependent hydroxylation of aliphatic C–H bonds by using two uncoupled metal sites. In spite of many investigations, including biochemical, chemical, and computational, details of the C–H bond oxygenation mechanism remain elusive. Herein we report an investigation of the mechanism of hydroxylation by PHM by

using hybrid quantum/classical potentials (i.e., QM/MM). Although previous investigations using hybrid QM/MM techniques were restricted to geometry optimizations, we have carried out ab initio molecular dynamics simulations in order to include the intrinsic flexibility of the active sites in the modeling protocol. The major finding

Keywords: ab initio calculations • copper • electron transfer • enzymes • molecular dynamics • reaction mechanisms

of this study is an extremely fast rebound step after the initial hydrogen-abstraction step promoted by the cupric-superoxide adduct. The hydrogen-abstraction/rebound sequence leads to the formation of an alkyl hydroperoxide intermediate. Long-range electron transfer from the remote copper site subsequently triggers its reduction to the hydroxylated substrate. We finally show two reactivity consequences inherent in the new mechanistic proposal, the investigation of which would provide a means to check its validity by experimental means.

Introduction

The exploitation of the oxidative power of dioxygen for the functionalization of aliphatic C–H bonds remains an objective of fundamental importance for the development of

profitable and environmentally friendly catalytic processes.^[1] In Nature, the family of noncoupled copper monooxygenases comprising peptidylglycine α -hydroxylating monooxygenase (PHM), dopamine β -monooxygenase (D β M), and tyramine β -monooxygenase (T β M) provides a fascinating source of inspiration.^[2–4] These ascorbate- and dioxygen-dependent copper enzymes catalyze the stereospecific hydroxylation of a C–H bond of their substrates through two magnetically uncoupled copper active sites, customarily referred to as Cu_M and Cu_H (see Scheme 1 for the reaction catalyzed by

Scheme 1. Stereospecific hydroxylation reaction catalyzed by PHM.

PHM).^[5] The two active sites are separated by a solvent-filled cleft with a width of around 10 Å and orchestrate the transfer of the four electrons and two protons required for substrate hydroxylation with concomitant release of H₂O. The imposing mass of biochemical data accumulated on these enzymes over the last three decades suggests that PHM, D β M, and T β M probably share a common mechanism with the chemistry taking place at the Cu_M site and the

[a] C. Meliá, Dr. S. Ferrer, Prof. V. Moliner
Departament de Química Física i Analítica
Universitat Jaume I, 12071 Castellón (Spain)
Fax: (+34)964-345654
E-mail: moliner@uji.es

[b] Dr. J. Řezáč
Institute of Organic Chemistry and Biochemistry
Academy of Sciences of the Czech Republic
Flemingovo nám. 2, 166 10 Prague 6 (Czech Republic)

[c] Dr. O. Parisel
Laboratoire de Chimie Théorique
UPMC, CNRS, UMR 7616, CC 137
4 Place Jussieu, 75252 Paris, Cedex 05 (France)

[d] Prof. O. Reinaud
Laboratoire de Chimie et de Biochimie Pharmacologiques
et Toxicologiques, Université Paris Descartes
PRES Sorbonne Paris Cité, CNRS UMR 8601
45 rue des Saints Pères, 75006 Paris (France)

[e] Dr. A. de la Lande
Laboratoire de Chimie-Physique, Université Paris Sud
CNRS, UMR 8000, 15, rue Jean Perrin
91405 Orsay CEDEX (France)
Fax: (+33)1-69-15-61-88
E-mail: aurelien.de-la-lande@u-psud.fr

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/chem.201301000>.

Cu_{II} site serving as an electron reservoir.^[5] Although the identification of the C–H bond-activating process is still debated,^[2,6,7] converging biochemical arguments support an enzymatic mechanism initiated by a hydrogen-abstraction step promoted by a cupric-superoxo ($[\text{Cu}_M^{\text{II}}(\text{O}_2^-)]^+$) adduct.^[2,8–10] Mononuclear biomimetic cupric-superoxo complexes have been characterized by a few groups,^[11–13] and some studies have also explored their oxidative behavior.^[14–21] These investigations on biomimetic systems thus provide support for a mechanism involving a hydrogen-abstraction step in the enzymes. On the other hand, the nature of the steps following the hydrogen abstraction is still highly debated and conflicting proposals have been advanced for the enzymatic mechanism (see, for example, Scheme 2 of ref. [6] or the Supplementary Information of refs. [20,26]). Computational approaches can advantageously complement experimental studies on enzymatic systems. In recent years, modeling based on quantum chemistry has, for example, confirmed the oxidative properties of cupric-superoxo complexes that were built as models of the enzymatic Cu_M active site^[7,22–24] or as models of biomimetic systems.^[25–27]

Hybrid quantum mechanics/molecular mechanics approaches have acquired an important role in the investigation of enzymatic mechanisms by computer simulations because they provide an efficient means to including the environment of the enzymatic active sites.^[28,29] Kamachi and Yoshizawa and co-workers^[23,24] were the first to successfully apply a density functional theory/molecular mechanics (DFT/MM) scheme to determine the equilibrium structures of the cupric-superoxo ($[\text{Cu}_M^{\text{II}}(\text{O}_2^-)]^+$) adducts within D β M and to determine the energy profile associated with hydrogen abstraction from the dopamine molecule. Crespo et al.^[7] shortly after reported a similar study with PHM. Both studies concluded that reaction pathways proceeding through a cupric-superoxo complex were unlikely and as an alternative proposed that a copper-oxo ($[\text{Cu}_M\text{O}]^{2+}$)^[7] or copper-oxyl ($[\text{Cu}_M^{\text{II}}\text{O}^\bullet]^{2+}$)^[24] species was responsible for the hydrogen-abstraction step. Such conclusions appear to be contradictory with experimental studies on PHM and D β M supporting the copper-superoxo ($[\text{Cu}_M^{\text{II}}(\text{O}_2^-)]^+$) hypothesis.^[2,9,10] We note that previous DFT and DFT/MM studies were based on geometry optimizations, therefore neglecting the dynamics of the protein. In addition, the evolution pathways for the product of the hydrogen-abstraction step might not have been sufficiently explored in previous studies. These elements provided motivation to investigate the cupric-superoxo hypothesis in more depth. As a result of the continuing development of efficient software and computer power, it is now possible to overcome some of the limitations of the previous computational methodologies. In this article we report an investigation of the PHM catalytic cycle based on hybrid DFT/MM Born–Oppenheimer molecular dynamics (BOMD) simulations.^[30] The BOMD methodology involves propagating the motion of the nuclei on the potential energy surface (PES) by application of Newton's laws. The forces acting on the nuclei are computed on-the-fly at the DFT/MM level. As a complement to geometry op-

timizations, this computationally intensive approach permits the inclusion of the intrinsic dynamics of the Cu_M active site in the modeling strategy. By using this technique it is, for example, possible to assess the stability of the optimized structures or to explore the conformational landscape visited by the system on the picosecond timescale. As described below, the molecular dynamics simulations have turned out to be crucial for revealing a rebound step following the initial hydrogen abstraction in PHM. Simulations of a long-range electron transfer (LRET) from the Cu_{II} active site also suggest that this is the final element necessary to complete the reaction. Overall, the results presented in this paper provide an updated view of the PHM catalytic cycle that is fully consistent with the large body of available experimental data.

Results

The details of the computational protocol can be found in the Computational Methods section of this article. The catalytic mechanism of PHM involves multiple steps starting from the binding of dioxygen to the Cu_M active site followed by attack of the C–H bond in the substrate and further evolution steps. In this work we successively investigated these reaction steps by using a combination of geometry optimizations and BOMD simulations. The Cu_{II} active site is composed of a copper ion coordinated by three histidine residues (107, 108, and 172). The coordination sphere of the Cu_M site includes the histidine residues 242 and 244 and the methionine residue 314. The substrate is docked in the vicinity of the Cu_M site (Figure 1). At the beginning of the substrate hydroxylation reaction both copper sites are in the formal +I state.

Dynamics of the cupric-superoxo adducts: Dioxygen binds to mononuclear copper centers through its π^* antibonding molecular orbital adopting either an end-on (η^1) or side-on (η^2) coordination mode.^[31,32] Chen and Solomon investigated the side-on coordination of dioxygen in a model of the PHM active site, but they did not consider the end-on coordination.^[5] Crespo et al. only investigated the end-on coordination in their DFT/MM study of PHM,^[7] whereas Kamachi et al. reported optimized geometries for both the side-on (singlet state) and end-on (singlet and triplet states) coordination modes in their DFT/MM modeling of D β M.^[23] Based on the energy differences between the different adducts, the authors concluded that the end-on coordination should be the most favorable coordination mode in the enzyme. In this work, as a first step, we performed geometry optimizations of the copper-dioxygen adducts in both the singlet and triplet spin states. The labeling of the important atoms for discussion is shown in Figure 1b. As reflected by the optimized inter-oxygen bond lengths ($d(\text{O}_\text{F}-\text{O}_\text{B}) = 1.27\text{--}1.32\text{ \AA}$), all these calculated adducts are typical cupric-superoxide complexes $[\text{Cu}_M^{\text{II}}(\text{O}_2^-)]^+$ (Table S1 in the Supporting Information).^[12,13] The DFT/MM-optimized geometries are in agreement with the fact that charge transfer towards dioxygen is

Figure 1. a) Image of the Cu_M active site of PHM with dioxygen coordinated end-on. The atoms treated at the QM level are shown as a ball-and-stick representation (101 atoms). The surrounding water molecules, which were treated at the MM level, are not shown for clarity. In the section “Dynamics of the cupric-superoxo adducts” the water molecules and the substrate were treated at the MM level. b) Schematic representation of the Cu_M active site after dioxygen binding: $[\text{Cu}_M^{\text{II}}(\text{O}_2^-); \text{SubH}_d]^+$ end-on adduct.

enhanced by side-on coordination in the singlet state.^[33] We note, however, that no structure of a side-on adduct could be optimized in the triplet state. When introducing dynamic effects by means of BOMD simulations, the singlet side-on adducts evolved towards end-on coordination on the subpicosecond timescale (see Figure S1 showing the evolution of the copper–oxygen distances during the BOMD simulation). Note that we attempted to stabilize the side-on coordination during the simulations by applying an harmonic constraint on the copper–oxygen distances, but the end-on coordination was systematically recovered when the constraint was released (see Figure S1). It is also instructive that when the DFT/MM-optimized structures were reoptimized in the gas phase and further relaxed by BOMD simulations, the coordination sphere of the copper ion adopts a two-coordination mode, expelling both the sulfur and dioxygen ligands (see Figure S2). Taken together, these computations indicate that the PHM backbone imposes constraints on the coordination sphere of the copper ion in a geometry enabling it to bind dioxygen. In addition, we can conclude that both spin states of the Cu_M active site stabilize only the end-on coordination. This conclusion is consistent with a previously reported crystal structure of aPHM crystallized with an inert substrate in which dioxygen binds end-on.^[34]

To the best of our knowledge, our study is the first to show the instability of the side-on coordination mode at the Cu_M site by means of BOMD simulations, in accord with the structure obtained by X-ray diffraction. The relative energies of the singlet and triplet adducts were evaluated by 2 ps BOMD simulations (see Figure S3 in the Supporting Information). The energy gap shows fluctuations of around 6 kcal mol⁻¹ in favor of the triplet state. These values are close to the estimates of Kamachi and Crespo for the optimized cupric-superoxo adducts in D β M and PHM, respectively.^[7,23] However, it is known that the singlet–triplet energy gap for copper–dioxygen adducts are extremely sensitive to the computational procedure, in particular, to the choice of exchange correlation functional.^[33,35] It is therefore not reason-

able to draw definitive conclusions about the most stable spin state of the adduct in PHM. For this reason we continued to consider the two spin states for this reactivity study. Our simulations reflect the great flexibility of the $[\text{Cu}_M^{\text{II}}(\text{O}_2^-)]^+$ core, as seen in the fluctuations of the O_2 –Cu–ligand angles (see Figure S4) in the 10 ps DFT/MM BOMD simulations regardless of the spin state. Remarkably, the time-dependent evolution of the O_2 –Cu–ligand torsion angle shows fast spinning of the distal oxygen atom around the Cu– O_p axes (see Figure S4). As a consequence, the distal oxygen atom and the substrate hydrogen atom (H_d) frequently face each other in conformations that are favorable for the reaction (see Figure S5).

Hydrogen-abstraction reaction: In this section we describe the oxidation of the substrate by the cupric-superoxide adducts. To this end, the list of atoms treated at the DFT level has been enlarged to include, in addition to the Cu_M active site (i.e., the copper cation, the His242, His244, and Met314 residues, and the superoxide), the entire substrate and nine water molecules surrounding the active site (Figure 1). We generated 15 structures by means of classical molecular dynamics (see the Computational Methods section for details). The 15 structures were further geometrically optimized at the DFT/MM level. The root-mean-square deviations (RMSDs) calculated for each of the optimized structures indicate that the positions of the protein atoms vary significantly between each structure (RMSD $\approx$ 2 Å), but that the main variations actually come from the positions and orientations of the water molecules surrounding the Cu_M site (see Figure S6 in the Supporting Information). Given the results of the previous section, we will now focus on the end-on coordination. Electron paramagnetic resonance (EPR) experiments on PHM suggested the formation of a diamagnetic $[\text{Cu}_M^{\text{II}}(\text{O}_2^-)]^+$ adduct, which means that the reaction probably occurs on the singlet surface.^[10,36] We have, however, also computed the energy profiles on the triplet potential energy surface. Some important geometrical parameters for the singlet state, such as the copper–ligand, inter-oxygen, and superoxide–substrate distances, are given in Table 1. Parameters characterizing the geometries of all the profiles can be found in Tables S2–S7 in the Supporting Information. The $d(\text{Cu}–\text{N}_{242})$ and $d(\text{Cu}–\text{N}_{244})$ bond lengths are almost identical in all the optimized structures (2.02–2.03 Å), whereas the $d(\text{Cu}–\text{S}_{314})$ bond length is longer (ranging from 2.31 to 2.39 Å) with an average value of 2.35 Å. The inter-oxygen bond length lies between 1.28 and 1.30 Å. To investigate the oxidative properties of these adducts, we selected the eight optimized structures in which the distance between the distal oxygen atom (O_d) and the substrate hydrogen atom (H_d) was below 2.8 Å. Constrained geometry optimizations along the reaction coordinate defined by $\xi = d(\text{C}_\alpha–\text{H}_d)–d(\text{O}_d–\text{H}_d)$ were carried out (see Figure 1b) to yield the energy profiles shown in Figure 2 and Figure S7.

To identify the nature of the chemical species transferred between the substrate and the superoxide ligand ($\text{H}^\bullet$ or H^-), we performed separate DFT/MM geometry optimizations of

Table 1. Variation of the characteristic distances along the hydrogen-abstraction step promoted by the end-on cupric-superoxide on the singlet surface. We report the average values and standard deviations (in parentheses) computed from the 15 $[\text{Cu}_2^{\text{II}}(\text{O}_2^-)\text{SubH}]^+$ adducts or the eight $[\text{Cu}_2^{\text{II}}(\text{O}_2^-)\text{H}\cdots\text{Sub}]^+$ and $[\text{Cu}_2^{\text{II}}(\text{OOH})\text{Sub}]^+$ structures. $[\text{Cu}_2^{\text{II}}(\text{O}_2^-)\text{H}\cdots\text{Sub}]^+$ corresponds to a value of $\xi \approx 0.4$ Å, that is, close to the maximum of the energy profiles shown in Figure 2. The average values for all the structures are given in the Supporting Information (Tables S2, S4, and S6).

	$[\text{Cu}_2^{\text{II}}(\text{O}_2^-)\text{SubH}]^+$ [Å]	$[\text{Cu}_2^{\text{II}}(\text{O}_2^-)\text{H}\cdots\text{Sub}]^+$ [Å] ($\xi \approx 0.4$ Å)	$[\text{Cu}_2^{\text{II}}(\text{OOH})\text{Sub}]^+$ [Å]
Cu ₂ center			
$d(\text{Cu}-\text{N}_{242})$	2.03 (0.01)	2.05 (0.02)	2.06 (0.02)
$d(\text{Cu}-\text{N}_{244})$	2.02 (0.02)	2.00 (0.02)	2.01 (0.03)
$d(\text{Cu}-\text{S}_{111})$	2.35 (0.02)	2.33 (0.03)	2.34 (0.03)
$d(\text{Cu}-\text{O}_6)$	2.01 (0.01)	1.96 (0.01)	1.96 (0.01)
$d(\text{O}_7-\text{O}_8)$	1.29 (0.01)	1.36 (0.00)	1.42 (0.01)
$d(\text{O}_7-\text{H}_4)$	2.98 (0.40)	1.13 (0.01)	1.00 (0.00)
$d(\text{C}_7-\text{H}_4)$	1.01 (0.00)	1.52 (0.01)	2.75 (0.40)
Substrate			
$d(\text{C}_7-\text{N})$	1.46 (0.00)	1.41 (0.00)	1.37 (0.00)
$d(\text{C}_7-\text{COO}^-)$	1.52 (0.00)	1.50 (0.01)	1.46 (0.00)
$d(\text{N}-\text{CO})$	1.35 (0.00)	1.37 (0.00)	1.38 (0.00)

Figure 2. a) Energy profiles of the hydrogen-abstraction step on the singlet energy surface. The last point of each profile corresponds to an unconstrained optimized structure. b) Influence of the exchange-correlation functional on the gas-phase energy profiles.

the carbocationic and carboradical substrate (respectively denoted as Sub^+ and $\text{Sub}^\bullet$). These geometry optimizations, in which only the substrate is included in the DFT partition, provided us with reference geometries for the $\text{Sub} < \mathbf{MI} >$ and Sub^+ entities. As seen in Figure 3, the bond lengths around the C_7 and $\text{N}(\text{H})$ atoms in the radical form are close to those found in the $[\text{Cu}_2(\text{OOH})\text{Sub}]^+$ intermediates (Table 1), but far from those found in the cationic form.

Figure 3. Evolution of three bond lengths on going from the cationic form (first line) to the radical form (second line) of a PHM substrate deprived of the H_4 atom. The third line corresponds to the distances obtained for the $[\text{Cu}_2(\text{OOH})\text{Sub}]^+$ intermediates (data taken from Table 1).

pending on the experimental conditions (nature of the substrate, pH, external reductants).^[37,38] It has also been shown that tunneling contributes to the process.^[39] Altogether, these experimental results establish the free energy of activation at around 13–15 kcal mol⁻¹.

The authors of ref. [7] obtained similar values as here by using the PBE functional. They concluded that the “inclusion of the whole protein, as in (their) calculations, imposes position constraints on the reactants that result in a higher activation energy”. Based on these numbers they ruled out the cupric-superoxo mechanism for PHM. Yoshizawa et al. used the B3LYP/MM approach to estimate the hydrogen-abstraction barrier from the dopamine substrate on the singlet surface within DβM, which yielded a value of around 23.1 kcal mol⁻¹.^[24] On the other hand, they obtained a barrier of 16.9 kcal mol⁻¹ in the gas phase.^[23] They also ruled out the cupric-superoxo mechanism for DβM.

It was thus important to examine whether the DFT/MM-computed values effectively rule out the cupric-superoxo hypothesis or whether they are partially biased by the com-

These results thereby indicate the formation of a radical intermediate ($[\text{Cu}_2^{\text{II}}(\text{OOH})\text{Sub}]^+$) and consequently that the oxidation of the substrate by the cupric-superoxide adduct can effectively be described as a hydrogen abstraction.

The activation potential energy barriers on the singlet surface range from 21–28 kcal mol⁻¹ with an average of 24.4 kcal mol⁻¹ and a standard deviation of 2.4 kcal mol⁻¹. The energy barriers are significantly higher on the triplet potential energy surface, ranging from 28–35 kcal mol⁻¹ (average: 31.6 kcal mol⁻¹, standard deviation: 2.3 kcal mol⁻¹, see Figure S7). At first glance these values seem to be rather large for an enzymatic reaction. The catalytic rate constant, k_{cat} , for PHM measured under steady-state conditions is in the order of 3–40 s⁻¹. However, the C_7-H bond cleavage is usually considered to be only partially rate-limiting.^[37,38] On the other hand, analysis of the intrinsic kinetic isotope effects provided estimates of the rate of C_7-H bond-breaking in the range of 810 ± 120 to 1330 ± 420 s⁻¹, de-

putational procedure. To analyze this question in more depth we recomputed the energies along one singlet energy profile and removed the electrostatic energy contributions arising from the presence of the protein and solvent. We took a singlet energy profile that showed an energy barrier of $23.5 \text{ kcal mol}^{-1}$. By using the above approach the gas-phase energy profile was significantly lowered by around 7 kcal mol^{-1} . Thus, the energy barrier is much closer to the experimental values. The larger energy barriers obtained at the DFT/MM level are thus essentially due to electrostatic interactions with the environment. We also mention that we tested the influence of the DFT exchange-correlation functional (OLYP, M06, B3LYP, O3LYP, PBE1PBE) on the energy barrier. For the type of chemical process investigated here, the PBE functional is found to compare rather well with other functionals like M06 or B3LYP (Figure 2b). We previously mentioned that the different starting structures differ essentially by the positions and orientations of the water molecules present in the PHM inter-domain space. From Figure 2, it can be seen that different orientations lead to variations in the energy barriers of several kcal mol^{-1} . Such variations are of the same order of magnitude as the polarization created by the environment. We thus conclude that the high DFT/MM barriers are not due to positional constraints imposed by the protein but rather to a lack of relaxation of the aqueous environment inherent to the DFT/MM geometry optimization procedure. To go further, a free-energy profile would have to be computed by using, for example, an umbrella sampling technique. To be carried out properly, such procedures require extensive conformational sampling of the environment, which is still very computer-time-consuming at the DFT/MM level. Nevertheless, we note that the list of atoms treated at the DFT level includes a significant layer of atoms surrounding the $\text{O}_p\text{--O}_d$ and $\text{C}_\alpha\text{--H}$ bonds, including nine water molecules, so that we can reasonably expect that the “gas-phase” energy profile already contains most of the energetics of the $\text{C}_\alpha\text{--H}$ bond cleavage. This qualitative agreement with the experimental data justifies a more in-depth investigation of the subsequent evolution pathways of the copper-hydroperoxide intermediate.

Evolution pathways of the copper-hydroperoxide intermediate: Our results are consistent with previous QM/MM studies regarding the formation

of the $[\text{Cu}_M^{\text{II}}(\text{--OOH}_2)\text{Sub}]^+$ intermediate through a hydrogen abstraction step. Various evolution pathways of this intermediate have been proposed in the literature based on either experimental or computational evidence.^[7,8,10,22] However, the intrinsic dynamics of the Cu_M active site were neglected in previous computational investigations. We have employed the BOMD technique to probe the existence of alternative reaction pathways that the $[\text{Cu}_M^{\text{II}}(\text{--OOH}_2)\text{Sub}]^+$ intermediate may follow on the singlet surface. As illustrated by the evolution of the $\text{C}_\alpha\text{--O}_p$ and Cu--O_p distances during the BOMD simulation (Figure 4a), we observe that the structure experiences an important chemical modification within less than 10 ps with the formation of a covalent bond between the substrate C_α atom and the proximal oxygen atom O_p , and the simultaneous decoordination of the O_p atom from the copper cation. Meanwhile, the $\text{O}_p\text{--O}_d$ bond length increases from 1.42 to 1.50 Å, which indicates that the peroxide moiety achieves a complete reduction. We also observe that the copper cation adopts a planar-trigonal geometry, a coordination that is consistent with a formal +I state. Summarizing these elements, the $[\text{Cu}_M^{\text{II}}(\text{OOH}_2)\text{Sub}]^+$ intermediate evolves within a few picoseconds towards an alkyl hydroperoxide intermediate $[\text{Cu}_M^{\text{I}}, \text{SubOOH}_2]^+$. Thus, the reaction event in the molecular dynamics simulation involves a radical recombination or, alternatively said, a rebound step, by analogy to the terminology found in the literature devoted to Cytochrome P450.^[40]

To ensure that the rebound step did not occur fortuitously in only one BOMD simulation, we repeated the simulations with four other optimized $[\text{Cu}_M^{\text{II}}(\text{--OOH}_2)\text{Sub}]^+$ complexes

Figure 4. a) Evolution of the $\text{C}_\alpha\text{--O}_p$ and Cu--O_p distances along five BOMD trajectories starting from distinct optimized $[\text{Cu}_M^{\text{II}}(\text{--OOH}_2)\text{Sub}]^+$ intermediates. The rebound event occurs at 4.7 ps, as seen by the sharp variation in distance. b) Similar BOMD simulations starting from different optimized structures of the $[\text{Cu}_M^{\text{II}}(\text{--OOH}_2)\text{Sub}]^+$ intermediate. The rebound occurs at 2.9, 5.2, 5.5, and 1.3 ps (from left to right).

chosen randomly among the eight optimized products of the hydrogen abstraction. The BOMD simulations were thus performed under different initial conditions (nuclear positions and velocities). The rebound was systematically observed during all the BOMD simulations within a few picoseconds (Figure 4b). This systematic evolution reflects the existence of a deep well on the potential energy surface, the origin of which is clearly related to the formation of a C_α – O_p covalent bond. Yet this thermodynamically favorable step requires large structural rearrangements within the active site: A rotation of the O_2H_2 group around the C_α – O_p axis and a shortening of the O_p – C_α distance so that both electrons can face each other and recombine, as illustrated in Figure 4a. Such structural rearrangements are, however, kinetically favorable thanks to the intrinsic flexibility of the Cu_M active site. Various pathways on the potential energy surface with no substantial energy barriers are certainly accessible. An analysis of the trajectories provides an explanation of the rapidity of the rebound: It only requires sufficient time for the hydroperoxide ligand to reorient so that C_α and O_p face each other. This timescale is mainly imposed by the rotation around the Cu – O_p bond, which occurs on the picosecond timescale. We also note that the absolute configuration of the C_α atom after the rebound is the same in all five BOMD simulations, in agreement with the stereospecificity of the PHM reaction.^[41]

Reduction of the alkyl hydroperoxide intermediate: We then modeled the long-range electron transfer (LRET) from the $Cu_{II}(I)$ active site. This non-adiabatic reaction has been the subject of various investigations in the past,^[22,42–44] but, to the best of our knowledge, none of these studies was based on the high-level DFT/MM approach. We restricted our investigation to the mechanistic proposals in which this event occurs after C–H bond cleavage.^[2] We considered two cases depending on whether the LRET takes place before and after the rebound step.

We first explored the structural consequences of the one-electron reduction of the $[Cu_M^{II}(\cdot OOH_\alpha), Sub]^+$ intermediate. To this end, we performed five BOMD simulations, as in the previous section, but with the addition of one electron in the DFT subsystem to mimic the electron transfer from the Cu_{II} active site. We observed important chemical modifications in the first 4 ps of almost each of the five BOMD simulations. We alternatively observed: 1) the reforming of the C_α – H_β bond, 2) the unbinding of the hydroperoxide ligand, 3) the protonation of the hydroperox-

ide ligand by a surrounding water molecule followed by the unbinding of oxygen peroxide, or 4) the loss of the coordination of His244 (see Figure S8 in the Supporting Information for details). The structure of the Cu_M site was stable in the fifth simulation. We attribute this large variety of chemical events to the instability of the reduced $[Cu_M^I(\cdot OOH_\alpha), Sub]^+$ intermediate. It seems suspicious that so many relaxation pathways may take place within an enzymatic mechanism. In fact, as we shall argue in the Discussion section, it appears more likely, from our point of view, that LRET occurs after the rebound.

To investigate the effect of the reduction of the $[Cu_M^I, SubOOH_\alpha]^+$ intermediate, we continued the BOMD simulations from the previous section for different times after the rebound step had occurred, but with a supplementary electron. The alkyl hydroperoxide intermediates spontaneously relax, cleaving the peroxide bond and leading to the formation of an alkoxide functional group on the substrate and a hydroxide ion (Figures 5a and S9 in the Supporting Information). After this initial phase the Cu_M active site adopts a square-pyramidal geometry within 2 ps with the inclusion of the substrate and the hydroxide ion in its coordination sphere. The axial position is occupied by Met314 or by one of the His242 or His244 residues (Figure 5b). Such geometrical rearrangements, which are systematically observed in all the simulations, must be the consequence of important modifications of the electronic structures after the electron injection. We note that the square-pyramidal geometry of the copper complex obtained after relaxation is consistent with a formal cupric state. We also observe that the increase in the O–O distance immediately after the injection of the electron is abrupt (Figure 5), which indicates that the peroxide π^* molecular orbital (MO) must have been filled during the process. A possible interpretation of the simulations is that reduction of the alkyl hydroperoxide intermediate induces 1) instantaneous ET from the Cu_M^I site

Figure 5. a) Evolution of the O_2 – O_2 distance upon addition of one electron during the BOMD simulation in the modeling of the initial structure 10 (see Figure S9 for similar graphs for the other structures). The vertical dotted line indicates the moment at which the rebound occurs in this simulation. Subsequently, one-electron reduction of the system was investigated at four different times (1.5, 2.0, 2.5 and 3 ps), the $[Cu_M^{II}(H_2O_2)(SubO_2)]$ intermediate forming after a short relaxation. b) Image of the $[Cu_M^{II}(H_2O_2)(SubO_2)]$ complex in a square-pyramidal geometry, here with the Met314 residue occupying the axial position.

to the π^* MO of the peroxide moiety (inner-sphere ET) and 2) cleavage of the peroxide bond due to the double occupation of the π^* MO. The formation of a $\text{SubO}_p \cdots \text{O}_d\text{H}$ pair implies a strong electrostatic repulsion between the two fragments that would account for the rapid increase in the O–O distance observed during the simulations (Figure 5a). It is particularly important to note that the rapid peroxide cleavage makes the LRET from the Cu_{H} irreversible. These *in silico* experiments indicate that the occurrence of LRET at the stage of the alkyl hydroperoxide intermediate leads to the final product from the PHM mechanism.

Discussion

Various proposals for the mechanism of action of noncoupled dicopper enzymes have been advanced over the last 30 years.^[3,4] Early proposals suggested that a two-electron input to bound dioxygen was necessary to obtain a species reactive enough for C–H bond cleavage. Species such as $[\text{Cu}_M^{\text{II}}(\text{OOH})]^+$ or $[\text{Cu}_M^{\text{II}}(\text{O}^*)]^+$ were proposed as reactants, thereby requiring one electron from each copper site before C–H cleavage. In the last decade an alternative hypothesis started to gain momentum with the works of Evans^[6] Prigge^[10] and Chen^[5,22] and their co-workers. These authors proposed the one-electron-reduced compound, $[\text{Cu}_M^{\text{II}}(\text{O}_2^*)]^+$, to be directly responsible for the C–H bond cleavage, giving rise to $[\text{Cu}_M^{\text{II}}(\text{OOH})]^+$ as an intermediate. From this intermediate, diverging evolution pathways were proposed (Scheme 2).

Klinman and co-workers proposed the LRET from the Cu_M^{I} active site to occur after the C–H bond activation leading to the formation of a radical substrate and a copper–oxo radical that can recombine to form the final products (Scheme 2, path a).^[8] To the best of our knowledge, no direct experimental or computational evaluation of the rate of this ET step (k_{ET}) in PHM have been reported. However, it has been argued, based on biochemical data,^[2,3,6] that k_{ET} is contained within the overall catalytic rate constant, k_{cat} , which is in the order of $3\text{--}40\text{ s}^{-1}$. The central question to

consider is to what extent the LRET may enter into competition with the rebound step that we expect to occur on the picosecond timescale? The computations reported herein do not provide an evaluation of the rate of this LRET. On the other hand, we may estimate an upper limit based on the following reasoning. In the most favorable situation, in which both the ET electronic states are nearly degenerate (in the framework of Marcus theory^[45] this would correspond to a situation in which the driving force for the ET, ΔG° , compensates the reorganization energy, λ , that is, $\Delta G^\circ = -\lambda$), the ET would take place without thermal activation and be limited by quantum tunneling only. In such a case the LRET rate essentially depends on the donor-to-acceptor distance.^[46] The copper-to-copper distance is around 11 Å. However, a value of 7 Å seems more reasonable to account for the nonzero size of the copper complexes and, eventually, for the possible occurrence of constructive interference among the ET tunneling pathways.^[47] According to ref. [48], if these optimum conditions were fulfilled, the LRET may eventually enter into competition with the rebound step. On the other hand, it seems rather unlikely that LRET would occur significantly faster than the rebound step. This possibility should motivate future investigations aiming at providing more quantitative estimates of the LRET rate. That said, we have shown that the injection of one electron into the $[\text{Cu}_M^{\text{II}}(\text{OOH})]^+$ intermediate is actually likely to lead to a highly unstable intermediate with many relaxation channels, not necessarily including the peroxide bond-breaking as proposed in path a of Scheme 2.

Chen and Solomon investigated a water-assisted OH transfer mechanism as a result of DFT computations on PHM active site models (Scheme 2, path b).^[5,22] The free energy for the coordination of water to $[\text{Cu}_M^{\text{II}}(\text{OOH})]^+$ was found to be almost zero ($-0.3\text{ kcal mol}^{-1}$), whereas the OH transfer step was found to be thermodynamically favorable by 10 kcal mol^{-1} . A similar mechanism was also proposed by Prigge et al.^[10] No estimate of the homolytic O–O bond-breaking energy barrier was reported by these authors, but a value of 9.7 kcal mol^{-1} was proposed by Kamachi et al. in the gas-phase modeling of the D β M mechanism.^[23] In our

BOMD simulations we never observed the coordination of water to the copper ion, nor the homolytic O–O bond-breaking, at least on the timescale sufficient for the rebound to occur.

Investigating the intramolecular oxygenation reaction within a $[\text{Cu-NMe-tren}]^+$ complex [tren = tris(aminoethyl)amine] by means of DFT modeling, de la Lande et al. proposed the formation of a cationic intermediate and a cuprous complex.^[26] A formal hydride transfer encompassing

Scheme 2. Evolution pathways for the cupric-hydroperoxide intermediate: Reductive cleavage (ref. [8]), water-assisted OH transfer (ref. [10] and [22]), inner-sphere ET (ref. [26]), or the rebound mechanism (this work).

both the hydrogen-abstraction step and this inner-sphere ET was proposed (Scheme 2, path c). However, the present simulations of the enzymatic active site show that such a mechanism is not relevant. This discrepancy probably arises from the fact that in the model complex, the substrate C–H bond was situated at the α position of an amino group instead of an amide as in the PHM substrate. Interestingly, however, we found in both studies that breaking the peroxide bond in a subsequent step can be triggered by the input of an electron from the Cu_{H} in PHM.

Comparing the rebound mechanism with the previous hypotheses, it is clear that such a radical recombination event is most probably the fastest evolution pathway for the cupric-hydroperoxide intermediate. Although such a hydrogen-abstraction/rebound sequence, promoted by a metal-superoxide adduct, has been advanced for other oxygenases, such as non-heme iron enzymes,^[49–51] it has rarely been proposed for noncoupled dicopper monooxygenases. Investigating the dioxygen activation by using biomimetic complexes, Reinaud and co-workers effectively suggested that a hydrogen-abstraction/rebound sequence with the formation of an alkyl hydroperoxide intermediate may be a plausible mechanism for noncoupled oxygenases.^[16,17] Our results for the PHM enzyme provide strong support for this mechanistic proposal. We draw attention to the considerable merits of the BOMD approach in revealing such reactive pathways of low activation energy when investigating the reactivity of metalloenzyme active sites. In the mechanism summarized in Scheme 3, dioxygen is activated upon coordination to the Cu_{M} site through a one-electron reduction step, similarly to the proposals of Prigge,^[10] Evans,^[6] and Chen^[22] and their co-workers. However, in the hydrogen-abstraction/rebound sequence, both $\text{C}_{\alpha}\text{--H}$ cleavage and $\text{C}_{\alpha}\text{--O}_{\text{p}}$ bond formation are achieved with no involvement of the remote Cu_{H} site. Subsequently, the LRET from the Cu_{H} site, simultaneously

with the oxidation of the Cu_{M} site, ensures the two-electron reduction of the alkyl hydroperoxide intermediate leading to the hydroxylated product. The Cu_{M} site has two successive and independent roles. First, it promotes dioxygenation of the substrate with a one-electron reduced dioxygen species with restoration of a formal +I oxidation state. Secondly, along with the Cu_{H} site, it acts an electron reservoir for the reduction of the resulting alkyl hydroperoxide intermediate. Given the complexity of the catalytic cycle of these metalloenzymes, so far it has been difficult to characterize experimentally the precise nature of most of the chemical intermediates. Indeed, apart from the emerging consensus of the cupric-superoxide as the promoter of the hydrogen-abstraction step, the chemical nature of the following intermediates remains elusive. In the mechanism we propose in Scheme 3, the two copper sites return to the +I redox state after the dioxygenation phase (hydrogen-abstraction and rebound steps). This characteristic of the mechanism opens the door to the design of experiments to test our hypothesis based on the present computer simulations. On one hand, it should in principle be possible to detect the accumulation of the alkyl hydroperoxide intermediate in the medium with enzymes deprived of a catalytically competent Cu_{H} active site. Despite a careful examination of the experimental literature devoted to catalysis by noncoupled monooxygenases, we have not found studies that have specifically addressed this possibility. Another hypothesis that might also be tested is the possibility of using PHM for reducing external alkyl hydroperoxide species to the hydroxylated equivalents, that is, shunting the hydrogen-abstraction and rebound steps. In other words, could PHM present alkyl hydroperoxide reductase activity?

Conclusion

The consensus has emerged over the last few years that a cupric-superoxide adduct, formed at the Cu_{M} active site, promotes the hydrogen-abstraction step in the catalytic cycle of noncoupled copper monooxygenases. The subsequent evolution of the cupric-hydroperoxide intermediate has been the subject of various contradictory mechanistic proposals. We have reported herein the first investigation of the PHM enzymatic cycle based on Born–Oppenheimer MD simulations at the DFT/MM level. With this powerful tool in hand we have been able to identify a rebound step occurring on the picosecond timescale immediately after the hydrogen abstraction. The rebound is very probably much faster than the alternative proposals (paths a–c in Scheme 2). The studies presented and discussed herein show that rebound provides an efficient means to trapping the product of the endergonic hydrogen-abstraction step in a state of lower energy with the formation of a C–O bond. We have proposed a new mechanism for the function of PHM that is fully compatible with the available experimental data on the enzyme (see, for example, ref. [2] for an up-to-date review on this point). In this mechanism, the two

Scheme 3. Proposed catalytic cycle accounting for the oxygenative properties of the noncoupled binuclear copper centers encountered in monooxygenases.

active sites return to the +I state after the oxidative phase (hydrogen-abstract and rebound steps) and are thus separated from the reductive phase (reduction of the alkyl hydroperoxide intermediate). This characteristic of the catalytic cycle, which is not encountered in the previously proposed mechanisms (Scheme 2), led us to generate two mechanistic hypotheses that should be amenable to experimental probes, therefore providing a means to check the validity of our proposals.

Computational Methods

We started with the crystal structure of the reduced PHM with bound substrate and dioxygen reported by Prigge et al. (PDB code 1SDW).^[34] Hydrogen atoms were added by using the HBUILD module of the CHARMM package^[52] (version c35) and the protein was solvated in a $80 \times 80 \times 55 \text{ \AA}^3$ box of flexible simple point-charge (SPC) water molecules (9895 water molecules).^[53] Five sodium cations were added to ensure electrical neutrality of the system. All the histidine residues were monoprotonated on the ND1 (HSD residue type) or HE1 atom (HSE residue type) depending on the interactions with the neighboring atoms identified in the 1SDW structure. Residues 235, 242, 244, 245, 279, and 305 were of the HSD type, whereas residues 107, 108, 172, 183, and 192 were of the HSE type. The simulated system contained a total 34516 atoms. The CHARMM 27 force field with CMAP corrections was used to treat the protein. The charges on copper ions were settled at 0.3 according to DFT calculations. The resulting charge transfer (0.7 au) was distributed on the ligand atoms. The van der Waals parameters for the copper ions ($\epsilon = 0.05 \text{ kJ mol}^{-1}$, $\sigma = 2.13 \text{ \AA}$) were taken from ref. [54]. The Brownian Langevin molecular dynamics simulation (MDS) using periodic boundary conditions and Ewald summation was carried out for 1.8 ns to relax the enzyme, keeping the Cu_H atom, residues His242, His244, and Met314, and dioxygen frozen to preserve the experimental coordination of O_2 . For the Cu_H site, harmonic restraints were applied to the copper–ligand distances by using the X-ray-determined equilibrium bond lengths and a force constant of $2000 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$. Fifteen snapshots regularly spaced in time were extracted and re-optimized to furnish the starting structures for the DFT/MM study.

Hybrid DFT/MM computations were realized in the Cuby program by coupling the DFT program deMon2k^[55] and the MM program CHARMM using a subtractive scheme with electrostatic embedding.^[56] The DFT/MM energy is computed according to Equation (1) in which $E_\text{Y}^\text{N-level}$ is the energy of the system Y (QM subsystem, isolated and in the environment) at the level of computation X (DFT or MM). Electrostatic embedding was achieved by the inclusion of the point charges of the classical atoms localized at distances less than $10 \text{ \AA}$ from the QM partition. To avoid energy double counting, the corresponding classical MM energy terms between these point charges and the atoms of the QM partitions were removed from $E_\text{SubSys+env}^\text{MM}$. Classical nonbonded interaction cutoffs used switching functions decaying from 1 to 0 between 14 and $16 \text{ \AA}$. The preliminary investigation of dioxygen coordination (described in the first paragraph of the Results section) was carried out with a QM partition that included the Cu_H atom, the His242, His244, and Met314 residues, and dioxygen (40 atoms). The substrate and the nine closest water molecules surrounding the dioxygen moieties were also included in the reactivity study (101 atoms). DFT computations were performed with a locally modified version of the deMon2k software by using the Perdew–Becke–Ernzerhof functional^[57] within the context of Auxiliary DFT.^[58] A polarized double-zeta atomic orbital basis set (DZVP-GGA) and a GEN-A2 auxiliary basis set were used on all atoms.^[59] Unrestricted formalism was employed for both triplet and singlet (broken symmetry) spin states. The DFT energies and gradients were corrected by using the empirical expression of Jurečka et al. to take into account dispersion effects (except for atom pairs including copper for which parameters are not available).^[60] QM/MM boundaries were treated with hydrogen link atoms, the

bond lengths of which had been previously optimized at the full DFT level. For some computations (see the main text) other exchange-correlation functionals, namely OPTX-LYP^[61,62] M06,^[63] B3LYP,^[64] O3LYP^[65] and PBE1PBE,^[57,66] were tested with the 6-311G** basis set.^[67,68] These tests were performed by using Gaussian 09.^[69]

$$E_\text{SubSys+env}^\text{DFT/MM} = E_\text{SubSys+env}^\text{MM} - E_\text{SubSys}^\text{MM} + E_\text{SubSys}^\text{DFT} \quad (1)$$

DFT/MM geometry optimizations were performed by keeping frozen the atoms localized at distances greater than $10 \text{ \AA}$ from the QM region. The reactants (the cupric-superoxide adducts) for the 15 starting structures were optimized. The energy profiles were calculated by using constrained geometry minimizations with application of an harmonic bias ($V_\text{bias} = k_\text{bias}(\xi - \xi_\text{target})^2$ with $k_\text{bias} = 500 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$) from $\xi = -1.6$ to $+1.5 \text{ \AA}$ (15 steps). We only determined the energy profiles of the eight reactants presenting an initial O_2 –H distance of less than $2.8 \text{ \AA}$ (i.e., $\xi > -1.8 \text{ \AA}$). The products were subsequently optimized without constraint. BOMD simulations were carried out in the canonical ensemble ($T = 300 \text{ K}$) by using a Nose–Hoover thermostat with a coupling time of 0.2 ps and an integration time step of 0.5 or 1 fs (for the 40 or 101 QM atoms systems, respectively). Atoms localized beyond $20 \text{ \AA}$ in the QM region were frozen in the BOMD simulations. DFT energies were fully converged at every BOMD step with tolerance criteria of 10^{-6} hartree with respect to the energy and of 10^{-3} for the density fitting coefficient error.^[55]

Molecular graphics (Figures 1, 4, and 5) were prepared by using VMD (version 1.8.6) and^[70] the graphics were prepared with Xmgrace.^[71]

Acknowledgements

This work was supported by Generalitat Valenciana (Spain, project PROMETEO/2009/053), the Ministerio de Economía y Competitividad (Spain, project no. CTQ2012-36253-C03-01) and the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic (research project RVO 61388963). A.L. is grateful to the Universitat Jaume I (Castelló, Spain) for travel funding (project no. INV-2011-50).

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Received: March 15, 2013

Revised: August 20, 2013

Published online: November 20, 2013

CHEMISTRY

A EUROPEAN JOURNAL

Supporting Information

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Investigation of the Hydroxylation Mechanism of Noncoupled Copper Oxygenases by Ab Initio Molecular Dynamics Simulations

Conchín Meliá,^[a] Silvia Ferrer,^[a] Jan Řezáč,^[b] Olivier Parisel,^[c] Olivia Reinaud,^[d]
Vicent Moliner,^{*,[a]} and Aurélien de la Lande^{*,[e]}

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SUPPORTING INFORMATION

	side-on (His 242)		side-on (Met 314)		end-on	
	S	T	S	T	S	T
d(Cu-O _p)	2.02	-	2.00	-	2.00 (2.172;0.077)	2.03 (2.088;0.130)
d(Cu-O _d)	2.09	-	2.08	-	2.83 (2.920;0.174)	2.88 (2.931;0.168)
d(O _p -O _d)	1.32	-	1.32	-	1.282 (1.275;0.021)	1.274 (1.274;0.027)
Cu-O _p -O _d	74.4	-	74.7	-	117.3 (114.1;11.2)	118.0 (120.3;11.0)
d(Cu-N ₂₄₂)	2.12	-	2.05	-	2.03 (2.223;0.058)	2.02 (2.058;0.085)
d(Cu-N ₂₄₄)	1.99	-	1.97	-	2.01 (2.223;0.063)	2.00 (2.040;0.068)
d(Cu-S ₃₁₄)	2.31	-	2.45	-	2.33 (2.471;0.077)	2.34 (2.390;0.142)
N ₂₄₂ -Cu-N ₂₄₄	106.7	-	106.9	-	112.7 (105.2;5.9)	113.6 (112.6;8.2)
N ₂₄₂ -Cu-S ₃₁₄	105.7	-	104.1	-	112.8 (120.3;8.8)	110.4 (113.8;8.1)
N ₂₄₄ -Cu-S ₃₁₄	106.1	-	105.4	-	108.3 (108.1;6.1)	111.9 (109.8;7.7)

Table S1: Geometrical characteristics of the $[\text{Cu}_M^{\text{II}}(\text{O}_2^*)]^+$ adducts in the singlet (S) and triplet (T) spin states. The labeling of the atoms is given on Figure 1. Two side-on structures are reported depending on the identity of the axial ligand (His242 or Met314). No triplet side-on adducts could be optimized. For end-on complexes the numbers in parentheses correspond to the average value and the standard deviations calculated from 10ps of BOMD simulations. All the distances are given in Å, the angles in degrees and the energies in kcal/mol.

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Struct. #	d(Cu-N ₂₄₂)	d(Cu-N ₂₄₄)	d(Cu-M ₁₁₄)	d(Cu-O ₉)	d(O ₉ -O ₉)	d(O ₉ -H ₁)	d(C ₉ -H ₁)	d(C ₉ -N)	d(C ₉ -COO)	d(N-CO)	ξ
1	2.027	2.023	2.342	2.017	1.28	2.528	1.111	1.466	1.533	1.355	1.417
2	2.028	1.984	2.381	2.009	1.29	2.602	1.112	1.46	1.529	1.348	1.49
3	2.042	2.049	2.312	2.016	1.28	2.889	1.112	1.449	1.521	1.346	1.777
4	2.013	2.033	2.330	2.001	1.293	3.382	1.115	1.454	1.528	1.352	2.267
5	2.026	2.020	2.333	2.004	1.281	3.048	1.110	1.454	1.519	1.346	1.938
6	2.025	2.044	2.311	2.031	1.284	2.725	1.113	1.455	1.521	1.345	1.612
7	2.016	2.041	2.353	2.004	1.297	3.577	1.115	1.456	1.521	1.350	2.462
8	2.031	1.999	2.331	2.017	1.285	2.449	1.113	1.458	1.522	1.351	1.336
9	2.021	2.014	2.355	2.014	1.282	2.79	1.110	1.460	1.523	1.34	1.680
10	2.009	2.006	2.343	2.011	1.287	2.701	1.110	1.460	1.527	1.348	1.591
11	2.033	2.040	2.370	2.002	1.294	3.618	1.111	1.455	1.526	1.350	2.507
12	2.053	2.006	2.357	2.000	1.288	3.079	1.109	1.453	1.516	1.357	1.970
13	2.024	2.022	2.340	1.999	1.288	2.619	1.111	1.457	1.526	1.355	1.508
14	2.033	2.013	2.376	2.009	1.286	3.508	1.115	1.460	1.527	1.346	2.393
15	2.033	2.010	2.388	2.003	1.291	3.255	1.110	1.452	1.521	1.346	2.145

Table S2: Geometrical characteristics of the initial $[\text{Cu}_9(\text{O}_9)]^+$ adducts (singlet end-on coordination) investigated in the reactivity study. All the distances are given in Å. The structures highlighted in orange are those selected for the optimization of the H-abstraction energy profiles (i.e. those for which $\xi > -1.8\text{Å}$).

Struct. #	d(Cu-N ₂₄₂)	d(Cu-N ₂₄₄)	d(Cu-M ₁₁₄)	d(Cu-O ₉)	d(O ₉ -O ₉)	d(O ₉ -H ₁)	d(C ₉ -H ₁)	d(C ₉ -N)	d(C ₉ -COO)	d(N-CO)
1	2.027	2.022	2.334	2.043	1.273	2.709	1.11	1.465	1.535	1.357
2	2.036	2.046	2.313	2.031	1.274	1.113	3.438	1.449	1.522	1.346
3	2.023	2.016	2.321	2.028	1.273	2.716	1.113	1.455	1.519	1.345
6	2.025	2.038	2.305	2.069	1.275	2.716	1.115	1.456	1.522	1.345
8	2.042	1.999	2.321	2.021	1.279	2.715	1.113	1.458	1.522	1.350
9	2.018	2.01	2.337	2.036	1.275	2.714	1.112	1.46	1.523	1.340
10	2.004	2.005	2.335	2.036	1.280	2.713	1.112	1.46	1.527	1.349
13	2.019	2.019	2.334	2.031	1.281	2.71	1.11	1.455	1.525	1.355

Table S3: Geometrical characteristics of the initial $[\text{Cu}_9(\text{O}_9)]^+$ adducts (triplet end-on coordination) investigated in the reactivity study. All the distances are given in Å. The structures highlighted in orange are those selected for the optimization of the H-abstraction energy profiles (i.e. those for which $\xi > -1.8\text{Å}$).

Struct. #	d(Cu-N ₂₄₂)	d(Cu-N ₂₄₄)	d(Cu-M ₁₁₄)	d(Cu-O ₉)	d(O ₉ -O ₉)	d(O ₉ -H ₁)	d(C ₉ -H ₁)	d(C ₉ -N)	d(C ₉ -COO)	d(N-CO)	ΔE
1	2.054	1.993	2.363	1.959	1.364	1.128	1.519	1.413	1.513	1.369	23.5
2	2.073	1.965	2.364	1.942	1.365	1.117	1.505	1.406	1.492	1.365	23.6
3	2.062	2.029	2.306	1.972	1.355	1.137	1.533	1.399	1.485	1.365	24.4
6	2.057	2.009	2.316	1.964	1.361	1.115	1.512	1.399	1.481	1.372	22.0
8	2.077	1.976	2.323	1.943	1.373	1.111	1.510	1.408	1.488	1.356	20.9
9	2.048	1.986	2.349	1.952	1.360	1.122	1.512	1.404	1.508	1.367	25.6
10	2.028	2.010	2.304	1.975	1.357	1.142	1.538	1.401	1.499	1.374	27.8
13	2.034	2.024	2.301	1.963	1.373	1.137	1.542	1.413	1.513	1.369	27.2

Table S4: Geometrical characteristics of the $[\text{Cu}_9(\text{O}_9)\dots\text{H}_2\dots\text{C}_9\text{a}]^+$ structures in the singlet state for $\xi \approx 0.4\text{Å}$, i.e. near the transition state of the H-abstraction step (see Figure 2). All distances are given in Å. The last column is the DFT/MM energy (kcal/mol) with respect to the initial optimized $[\text{Cu}_9(\text{O}_9)]^+$ adduct.

Struct. #	d(Cu-N ₂₄₂)	d(Cu-N ₂₄₄)	d(Cu-M ₁₁₄)	d(Cu-O ₉)	d(O ₉ -O ₉)	d(O ₉ -H ₁)	d(C ₉ -H ₁)	d(C ₉ -N)	d(C ₉ -COO)	d(N-CO)	ΔE
1	2.053	2.002	2.333	1.977	1.351	1.142	1.526	1.416	1.512	1.368	29.3
2	2.051	2.026	2.313	1.964	1.343	1.189	1.56	1.413	1.503	1.367	32.5
3	2.067	1.998	2.347	1.950	1.414	1.012	2.427	1.370	1.450	1.366	34.5
6	2.019	2.036	2.314	2.002	1.339	1.151	1.536	1.409	1.489	1.359	30.2
8	2.053	1.987	2.323	1.972	1.348	1.135	1.519	1.410	1.484	1.365	27.9
9	2.039	1.994	2.348	1.980	1.345	1.132	1.513	1.413	1.491	1.353	31.0
10	2.013	2.016	2.339	1.975	1.349	1.149	1.534	1.413	1.506	1.362	33.5
13	2.015	2.031	2.308	1.964	1.361	1.147	1.544	1.406	1.501	1.373	33.5

Table S5: Geometrical characteristics of the $[\text{Cu}_9(\text{O}_9)\dots\text{H}_2\dots\text{C}_9\text{a}]^+$ structures in the triplet state for $\xi \approx 0.4\text{Å}$, i.e. near the transition state of the H-abstraction step (see Figure 2). All distances are given in Å. The last column is the DFT/MM energy (kcal/mol) with respect to the initial optimized $[\text{Cu}_9(\text{O}_9)]^+$ adduct.

Struct. #	d(Cu-N ₂₄₂)	d(Cu-N ₂₄₄)	d(Cu-M ₃₁₄)	d(Cu-O _a)	d(O _p -O _d)	d(O _p -H _a)	d(C _p -H _a)	d(C _p -N)	d(C _p -COO')	d(N-CO)
1	2.056	1.994	2.379	1.937	1.420	0.992	2.389	1.376	1.477	1.379
2	2.077	1.974	2.383	1.950	1.424	0.996	2.603	1.369	1.459	1.370
3	2.069	2.058	2.307	1.964	1.413	0.997	3.218	1.366	1.453	1.375
6	2.046	2.028	2.336	1.972	1.400	0.999	3.538	1.370	1.456	1.369
8	2.069	1.991	2.341	1.944	1.428	1.020	2.504	1.368	1.449	1.384
9	2.060	2.000	2.365	1.951	1.428	0.999	2.611	1.37	1.457	1.367
10	2.079	1.998	2.297	1.966	1.408	0.993	2.606	1.365	1.467	1.378
13	2.026	2.019	2.333	1.959	1.435	0.992	2.520	1.367	1.465	1.385

Table S6: Geometrical characteristics of the optimized $[\text{Cu}_2^{\text{II}}(\text{OOH})_2\text{Sub}]^+$ intermediates after H-abstraction in the singlet state. All distances are given in Å. The structures highlighted in blue are those selected as starting geometries for BOMD simulations (see Figure 4).

Struct. #	d(Cu-N ₂₄₂)	d(Cu-N ₂₄₄)	d(Cu-M ₃₁₄)	d(Cu-O _a)	d(O _p -O _d)	d(O _p -H _a)	d(C _p -H _a)	d(C _p -N)	d(C _p -COO')	d(N-CO)
1	2.072	2.01	2.362	1.949	1.430	1.008	2.425	1.378	1.476	1.373
2	2.082	2.03	2.296	1.986	1.402	0.987	2.329	1.367	1.447	1.375
3	2.051	2.002	2.316	2.002	1.339	1.140	1.521	1.408	1.491	1.358
6	2.041	2.035	2.316	1.955	1.408	0.979	2.367	1.371	1.460	1.374
8	2.066	1.992	2.343	1.942	1.424	1.001	2.408	1.369	1.452	1.382
9	2.057	1.999	2.366	1.948	1.426	0.997	2.403	1.370	1.460	1.368
10	2.039	2.033	2.304	1.970	1.401	1.020	2.432	1.371	1.464	1.372
13	2.015	2.028	2.342	1.950	1.429	0.997	2.401	1.371	1.466	1.383

Table S7: Geometrical characteristics of the optimized $[\text{Cu}_2^{\text{II}}(\text{OOH})_2\text{Sub}]^+$ intermediates after H-abstraction in the triplet state. All distances are given in Å. The structures highlighted in blue are those selected as starting geometries for BOMD simulations (see text and Figure S6).

Figure S1: Time evolution of the Cu-O_a and Cu-O_p distances in four BOMD simulations. yellow and blue curves: two free BOMD simulations starting from two side-on geometries where the axial ligand is either the His242 residue (yellow) or the the Met314 residue (blue). Green lines: BOMD simulation with a preliminary relaxation phase where the internal geometry of the Cu-O_p-O_a core is frozen during 2.75 ps. When the Cu-O_p-O_a are free to move again (at 2.75 ps), the Cu-O_a distance fluctuates around 3 Å, reflecting the adoption of the end-on coordination mode. Red lines: BOMD simulation with application of an harmonic constraining potential on the difference between the two Cu-O_a distances ($V_{\text{bias}} = k_{\text{bias}} [d(\text{Cu-O}_a) - d(\text{Cu-O}_p)]^2$). A force constant of $k_{\text{bias}} = 300$ (kcal/mol)/Å² is chosen for the 0-2.5 ps time window. The side-on coordination is associated to a weak O₂ binding as testified by the large Cu-O_{p/d} distances (2.30 Å). The biasing potential is weaker in the 2.5-2.75 ps time window ($k_{\text{bias}} = 50$ (kcal/mol)/Å²). Finally when $k_{\text{bias}} = 0$ (no constraints) after 2.75 ps, the Cu-O_a distance starts fluctuating around 3 Å, reflecting again the adoption of the end-on coordination mode.

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Figure S2: mechanical effect of the PHM backbone on the coordination sphere of the Cu_M site, here in the case of the triplet $[\text{Cu}_M^{\text{II}}(\text{O}_2^{\cdot-})]^+$ adduct: when the DFT/MM optimized structure is relaxed in the gas phase through geometry re-optimization or BOMD simulation the coordination sphere experiences dramatic changes.

Figure S3: Time evolution of the singlet-triplet energy gap (in kcal/mol) along 2 ps of BOMD simulation on the singlet potential energy surface.

Figure S4: BOMD simulations of the $[\text{Cu}_M^{\text{II}}(\text{O}_2^+)]^+$ adducts. Left: evolution of the valence angles involving the proximal oxygen atom (O_pCuX). The numbers on the graph correspond to the average values and to the standard deviations. Right: Variations of the torsion angles $\text{O}_d\text{O}_p\text{CuX}$ ($\text{X}=\text{N}242$ in blue, $\text{X}=\text{N}244$ in red, $\text{X}=\text{S}314$ in green). Upper panels for the singlet adduct; lower panels for the triplet adduct

Figure S5: Evolution of the $\text{O}_d\text{-H}_S$ distance during the BOMD simulation of the singlet end-on $[\text{Cu}_M^{\text{II}}(\text{O}_2^+)]^+$ adduct.

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Figure S6: RMSD for the heavy atoms of the protein and for the water molecules within the interdomain cavity between for each of the extracted from the classical MD simulation and further re-optimized at the DFT/MM level. RMSD are computed with respect to structure 7.

Figure S7: energy profiles of the H-abstraction step on the triplet energy surface.

Figure S8: BOMD simulations of the $[\text{Cu}_M^{\text{I}}(\text{OOH})_2, \text{Sub}']$ intermediate with special emphasis on the most interesting inter-atomic distances. The starting geometries are the same as on Figure 4 but with different initial velocities. Top-Left: in this simulation the $\text{C}_\alpha\text{-H}_\delta$ is reformed at 3.6ps as seen with concomitant breaking of the $\text{O}_\delta\text{-H}_\delta$ bond leading to a $[\text{Cu}_M^{\text{I}}(\text{O}_2^-), \text{SubH}_\delta]$. Top-Right: In this simulation the hydroperoxide ligand unbinds from the copper center after 2.5ps. Bottom-Left: in this simulation the hydroperoxide ligand is protonated by a surrounding water molecule (1.7 ps), which subsequent unbinding of H_2O_2 from the Cu_M^{I} center (1.9ps). Bottom-Right: in this simulation the coordination sphere of the Cu_M ions is denaturated with decoordination of His244 and coordination of the C_α carbon. No chemical event occurred during the 4ps of the fifth BOMD simulation (data not shown).

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Figure S9: O-O bond cleavage as a systematic consequence of the addition of one electron into the $[\text{Cu}_M^I, \text{SubOOH}_n]^+$ intermediate. Top-left: In black $\text{O}_p\text{-O}_d$ distance along the BOMD trajectory of the $[\text{Cu}_M^{\text{II}}(\text{OOH}_n), \text{Sub}]^+ / [\text{Cu}_M^I, \text{SubOOH}_n]^+$ intermediate (RR occurs at 1.5 ps). In color, same distance after addition of one electron: the peroxide bond spontaneously breaks. Top-right: similar graphics for the four other BOMD simulations along which RR was monitored (see Figure 2). Bottom: Four molecular snapshots after O-O bond cleavage and relaxation. Only the atoms treated at the DFT level are shown. These pictures illustrate the adoption of a square pyramidal geometry around the copper atom. The axial position is occupied either by Met314 or by one of the His242 or His244 residues.

8. Summary and Outlook

8. Summary and Outlook

In accordance with the original objectives, different studies have been presented in this PhD Thesis. The main conclusions drawn from this research can be summarized as follows:

Hydrolysis of a β -lactam antibiotic, CEF, was first studied *in vacuo* including the full molecule and a water molecule as a nucleophilic agent, followed by the counterpart study in aqueous solution by means of hybrid QM/MM potentials. The results allow concluding that:

- The inclusion of the full molecule of the CEF in the gas phase molecular model has been demonstrated to be crucial since its carboxylate group can activate the nucleophilic water molecule.
- The flexibility of the substrate implies the existence of a huge number of possible conformers, some of them implying formation of intramolecular hydrogen bond interaction that can determine the energetics of the conformers defining the different states along the reaction profile.
- From a computational point of view, the results show that PM3 semiempirical Hamiltonian provides results that are in qualitative agreement with higher level calculations based on DFT; B3LYP and M06-2X hybrid functional used with standard 6-31G* basis set.
- The PMFs computed at PM3/MM level and corrected with the B3LYP and M06-2X functional, show a step-wise mechanism which is kinetically determined by the nucleophilic attack of a water molecule activated by the proton transfer to the carboxylate group of the substrate.

Hybrid QM/MM MD simulations have been performed to explore the mechanisms of hydrolysis of two antibiotics, IMI and CEF, catalyzed by a mono-nuclear β -lactamase, CphA from *A.hydrophila*. The main conclusions of this study are:

- The hydrolysis of the four membered ring of β -lactam antibiotics takes place through a concerted mechanism where the nucleophilic attack on the carbonyl group of the β -lactam ring, the protonation of the N atom and scission of the carbon-nitrogen bond take place in a single chemical step.
- Electronically different transition state structures are revealed in a deeper comparative analysis: while the TS of the CEF is a ionic species with negative charge on nitrogen, the IMI TS presents a tetrahedral-like character with negative charge on oxygen atom of the carbonyl group of the lactam ring. Dramatic conformational changes can take

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place in the cavity of CphA to accommodate different substrates, which would be the origin of its substrate promiscuity.

- From the energetic point of view, including the correction at M06-2X-MM level, render free energy barriers of 32.6 and 35.6 kcal/mol for the hydrolysis of IMI and CEF, respectively. This trend would be in agreement with the fact that CphA shows only activity against carbapenem antibiotic.

Regarding the second block of this PhD Thesis this study represents the first investigation of the PHM enzymatic cycle based on Born-Oppenheimer MD simulations at the DFT/MM level. The conclusions are summarized as follows:

- A rebound step has been identified occurring on the picoseconds timescale immediately after the hydrogen abstraction. The rebound is very probably much faster than the alternative proposals.
- The rebound provides an efficient means to tapping the product of the endergonic hydrogen-abstraction step in a state of lower energy with the formation of a C-O bond.
- The two active sites return to the +I state after the oxidative phase and are thus separated from the reductive phase.

The work done in this PhD Thesis offers many possibilities for future research. It opens the doors to the deep study of the mechanism of enzymatic reaction to know the origin of the selectivity of the different MβLs, and as a consequence, the discovery of specific and potent inhibitors of these enzymes against the pathogenic bacteria.

In the group of copper monooxygenase enzymes, the question of the activation of molecular oxygen is an open question of debate in the fields of biochemistry and chemistry. There are many contradictory reaction mechanism proposals, the direction of future studies could be, together with experimental evidence supporting the theoretical results, the study of such reaction mechanisms.

Through the use of the QM/MM hybrid methodology, the study of biological systems can be deepened, obtaining results that can be compared with available experimental results and to guide the design and synthesis of new high value chemicals. Nevertheless, methodological developments are required in order to describe the complex biological systems (i.e. those active sites involving transition metals) at high accuracy.

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