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COOL BIOPHYSICS

Simulating Enzyme Catalysis

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Chemistry is about transformations between compounds as the result of forming and breaking bonds between their atoms. A detailed knowledge of these processes should open the door to one of the most desired goals in this field, which is the control of the rate constants that govern the time dependence of the concentrations of reactants and products. Changes in the concentration and preparation of the reactants, the nature of the solvents and external conditions (such as pressure and temperature) can be employed to speed up or slow down a chemical reaction. One of the major breakthroughs in the field of chemical kinetics was achieved when it was recognized that certain substances, *the catalysts*, were able to accelerate chemical reactions without being consumed during the process.

Living organisms use catalysts, known as enzymes, to accelerate chemical processes making them to take place in timescales compatible with life. The first observation of enzyme activity dates to the end of XVIII century. Amylase was the first enzyme to be isolated and urease was the first one to be identified as being a protein. Enzymes achieve rate enhancements as large as 1020 relative to the counterpart uncatalyzed reaction [1]. In addition, they present high efficiency, selectivity, few unwanted side products, and they usually work at aqueous mild conditions of pH, temperature or pressure, which converts them in very attractive catalysts for industrial purposes [2]. Nowadays we know that most of *biocatalysts* are proteins and the characteristics of these biomacromolecules, as discussed in this contribution, are responsible of their amazing catalytic properties. Many efforts have been devoted during the last years to understand the mechanism of action of these catalysts and to the practical implementation of this knowledge into the development of new biocatalysts.

The catalytic cycle of an enzyme involves at least three steps: substrate binding, chemical transformation and product release. The simplest kinetic scheme used to understand enzyme catalysis is that proposed by Michaelis and Menten, which involves the formation of a substrate-

catalyst complex previous to the actual chemical reaction:

$$E + S \rightleftarrows ES \longrightarrow P + E \quad (1)$$

We will focus here on the chemical step, showing how enzymatic chemical reactions can be analysed from a computational perspective and what have we learnt from these studies. We discuss theoretical approaches for the study of catalytic activity, consensus and discrepancies reached until now to describe the origin of this activity, and attempts to use all these findings in the development of new and more powerful catalysts.

Enzyme catalysis in Theoretical Chemistry

A complete understanding of chemical reactions requires an interpretation of the macroscopic observations (in this case the rate constant of the chemical step) from a microscopic perspective. The *Transition State Theory* (*TST*) is probably the most used theory to predict and interpret macroscopic rate constants from first principles [**3**]. TST establishes that the rate of a chemical transformation can be expressed as the probability to reach an activated conformation (or Transition State, TS) from the reactants (or Michaelis complex, ES in (1)) multiplied by the frequency needed for this complex to achieve the product state. The probability that reactants reach the activated conformation is given by the free energy difference with the TS, or activation free energy ($\Delta G^{\ddagger}$). The TST expression for the rate constant k_r of an unimolecular reaction (as the conversion from ES to P reflected in (1)) is then given by:

$$k_r = \frac{k_B T}{h} e^{-\frac{\Delta G^{\ddagger}}{RT}} \quad (2)$$

where the exponential factor accounts for the probability to achieve the TS and the preexponential term (where k_B is the Boltzmann constant, h is Planck's constant and T is temperature) is the universal frequency term. This theory is valid for many of the cases in which chemists are interested, except for those situations where thermal equilibrium is not reached or when light nuclei (hydrogen) are transferred. Nevertheless, even in this latter case, the TST expression can be corrected to account for those situations in which the light particle can tunnel through the energy barrier due to its non-classical nature.

The application of this expression to gas phase reactions of small molecules is quite straightforward. Chemical reactions are about electron reorganization which means that *quantum mechanics* (*QM*) is required. QM methods can then be used to obtain the energy of the molecules as a function of different coordinates that drive the system from the reactant state to the product state. For example, in a simple S_N2 reaction such, as $OH^- + CH_3Cl \rightarrow CH_3OH + Cl^-$ the distances associated to the bond to be broken ($C-Cl$) and the bond to be formed ($O-C$) can be used to obtain the potential energy of the system during the chemical transformation. Once the potential energy surface (PES) of the reaction is known (see **Figure 1a**) the free energies associated to the reactants and to the TS (and then the activation free energy) can be easily obtained using Statistical Thermodynamics under useful approximations, such as the harmonic behaviour of

molecular vibrations.

However, the picture becomes much more complicated when dealing with enzymatic reactions. First, the molecules under study are much larger, preventing the QM description of the full system. A realistic study of an enzymatic reaction must consider not only the substrate and the residues in the *active site*, but also the whole protein, possible cofactors, solvent molecules and ions. Typically, a realistic system designed to analyze an enzymatic reaction may easily contain thousands of atoms. Moreover, these studies must afford an additional problem: the large number of degrees of freedom of the system. The resulting PES (**Figure 1b**) is very rough, containing a hierarchy of valleys and sub-valleys of conformations that can be populated or not depending of the conditions in which the experiment is performed. The protein is not a rigid scaffold where the reaction takes place. Instead, the protein structure may suffer some changes during the reaction and the proper protein conformation must be chosen to obtain the right picture of the process. Thus the study of enzymatic reactions clearly requires the development of new methodologies, able to solve the problems associated to the size and conformational diversity of enzymes.

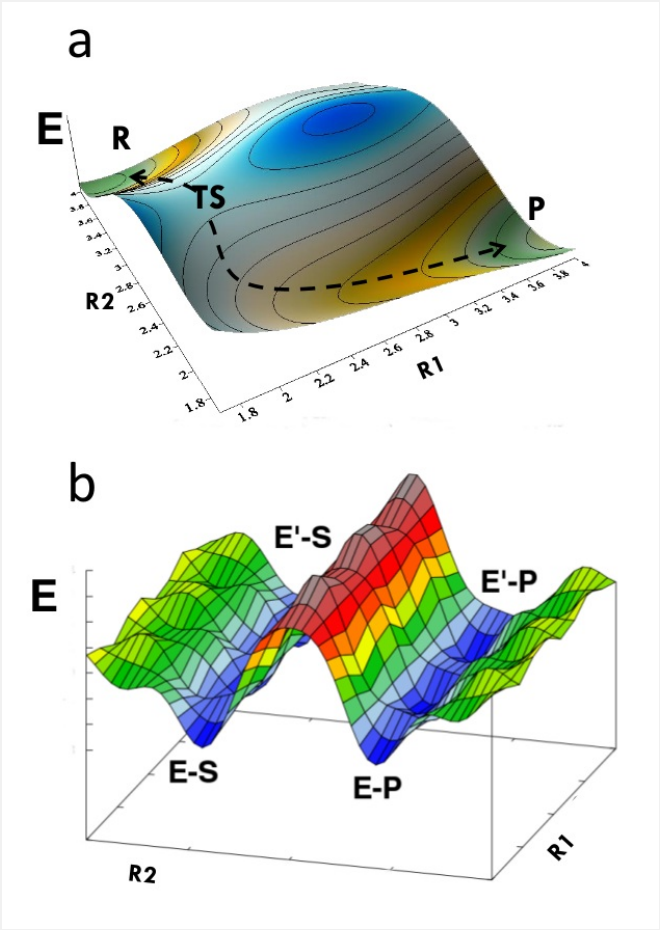

Figure 1. Potential Energy Surface corresponding to a chemical reaction. (a) *Small molecular system.* ***(b)*** *Enzyme. In large biomolecular systems there are myriads of possible stationary structures and the corresponding Potential Energy Surface is very rough. Simulations methods are needed to explore the relevant conformations of the system.*

New problems require new strategies

The 2013 Nobel Prize in Chemistry was awarded to Martin Karplus, Michael Levitt and Arieh Warshel for the development of multiscale models for complex chemical systems. Their pioneering work during the 70's [**4**,**5**] paved the way for modern computational strategies, allowing the study of chemical reactions in condensed media. The basic idea behind current methods is a partitioning of the whole system into two subsystems. The first one is described using QM methods and comprises all the atoms involved in the chemical reaction and then those atoms whose electrons must be explicitly described. The remaining part of the system is described using *Molecular Mechanics* (*MM*) interacting with the QM subsystem (**Figure 2a**). The resulting combination of methodologies is usually refereed to as *QM/MM hybrid methods* and takes advantage of both, the

reliability of quantum mechanics to describe bond breaking/forming processes and the efficiency of MM methods to evaluate the energy associated to conformational changes in the rest of the system.

QM/MM hybrid Hamiltonians provide the energy of the system. These methods can then be combined with Molecular Dynamics (MD) simulations in order to explore different configurations of the system. Different computational techniques, such as Umbrella Sampling, Metadynamics, Thermodynamic integration and others can then be employed to extract the variation of the free energy of the system when the reaction advances. These free energy profiles (**Figure 2b**) inform about the spontaneity of the process (the reaction free energy) and the rate (the activation free energy, see eq. (2)). This combination of methodologies, often refereed as QM/MM MD simulations, have opened the way to a vast number of studies of enzyme reactions which have been useful to elucidate a large number of reaction mechanisms and the role of specific enzyme residues in the catalysis [**6**]. This knowledge can then be used to rationalize, or even predict, the consequences of mutations on catalysis, which in turn can guide the design of new biocatalysts, as discussed below.

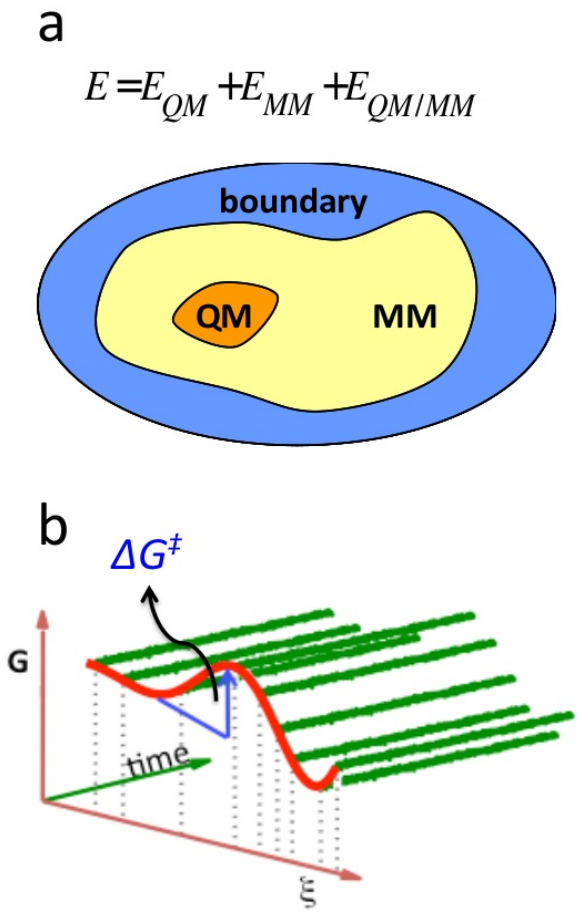

Figure 2. QM/MM methods for computational simulations of enzyme catalysis (a) *In hybrid QM/MM methods the total system is divided into a small region described at the QM level, where the chemical process of interest takes place, and the surroundings, described at the MM level. The total energy is the sum of the energy of the subsystems (E_{QM} and E_{MM}) and the interaction energy ($E_{QM/MM}$).* ***(b)*** *Molecular simulations can be carried out to obtain the free energy associate to changes along a particular coordinate. If this coordinate drives the system from reactants to products the activation free energy determines the rate of the process.*

Models to explain enzyme catalysis

Theoretical simulations of enzyme catalysis have been used to dissect the origin of the catalytic efficiency of enzymes. Nowadays, a growing consensus in the community is being reached around the seminal idea of Pauling [**7**], who assumed the complementarity between the enzyme's active site and the TS structure, originally expressed in terms of the *lock and key* analogy. The enzyme stabilizes more the TS than the Michaelis complex and thus the activation free energy appearing in eq. (2) would be lower than in the absence of the catalysts. Warshel and

co-workers [8,9] reformulated and quantified this hypothesis showing that the TS stabilization is basically due to the electrostatic environment provided by the active site of the enzyme. According to these authors, the active site displays an electrostatic environment prepared to accommodate the charge distribution of the reacting system at the TS. This provides a strong stabilization of the TS, relative to the reactants, without changing the enzymatic environment too much during the chemical transformation of the substrate. In contrast, in aqueous solution, water molecules can adapt to the reaction charge flow but, in most of the cases, an energy penalty, the reorganization energy, should be paid to rearrange the solvent molecules. These differences between the enzymatic and non-enzymatic reactions are qualitatively illustrated in **Figure 3**. The preorganization of the enzyme active site, which is a consequence of its tridimensional covalent structure, avoids the energy cost that must be paid in the uncatalyzed reaction to reaccommodate the solvent, lowering the activation free energy and increasing the rate of the process.

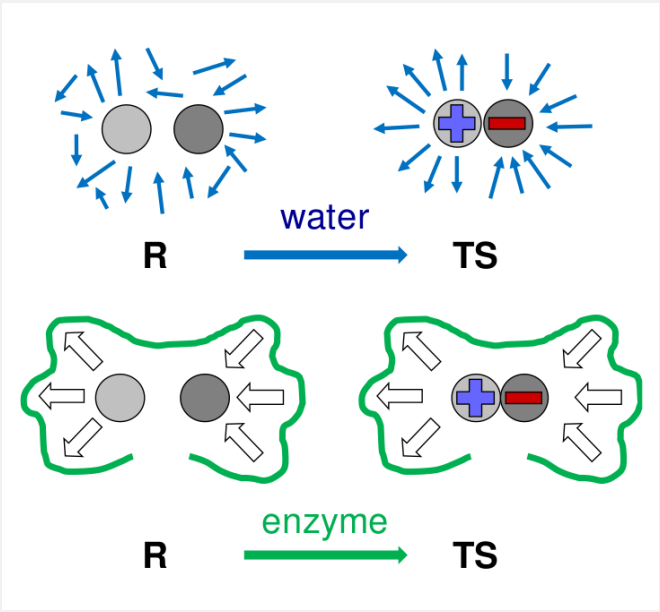

Figure 3. Reorganization around the substrate in the water solvent and in the active site of an enzyme. Top: *When a chemical reaction takes place in aqueous solution, water dipoles need to be reoriented to accommodate to the new charge distribution. The substrate is represented as spheres while water dipoles are represented by arrows.* ***Bottom:*** *The active site of an enzyme is preorganized to stabilize the charge distribution of the reaction Transition State, avoiding a free energy penalty and thus increasing the reaction rate. Arrows represent electrostatic interactions of the substrate with active site residues.*

Nevertheless, other theories have been invoked to explain the origin of the catalytic efficiency of enzymes. Some of the most popular proposals during previous years where those emphasizing the role of the enzyme in the formation of the Michaelis complex (ES in (1)). While the counterpart reaction in solution would require to approach and orient conveniently the reacting fragments, with a concomitant free energy cost, this rearrangement of the reacting fragments would occur in the enzyme already at the Michaelis complex and its free energy cost would be subsumed in the binding free energy. Thus, the key for the catalysis now focuses on the rearrangement of the substrate that takes place favourably in the enzyme active site and has an energetic cost in solution. In this sense Bruice et al. [10] introduced the concept of *Near Attack Conformations* (NAC), which are ground state conformers that closely resemble the TS. NACs would be as turnstiles through which the ground state must pass to reach the TS. According to this idea, the enzyme active site would decrease the activation free energy by increasing the probability of finding NAC-like structures.

Other explanations have been also invoked to explain enzyme catalysis. In the case of proton or hydride transfer reactions it has been suggested that enzymes could favour the transfer of these light particles by quantum tunnel behaviour, avoiding the necessity to overcome the energy barrier that is assumed in classical mechanics. Other authors have insisted in the role of coupling of protein vibrations with the chemical subsystem, in such a way that these vibrations could push the reacting system uphill along the energy barrier. However, when quantified with adequate simulations all these effects were shown to contribute very modestly, if any, to catalysis. [11–13]. The TS stabilization appears to be the major source for catalysis [14]. This idea can be then used to understand the role of protein mutations on the rate constant and to guide the design of new biocatalysts.

Nowadays there is a long list of enzymes that have been thoroughly investigated by means of flexible QM/MM techniques. These studies have been useful, not only to establish the corresponding reaction molecular mechanisms, but also, and probably more importantly, to investigate the principles of enzyme catalysis.

An illustrative example: the Catechol O-Methyl Transferase

The enzyme *catechol O-methyl transferase* (COMT) is an excellent prototypic system that has been the subject of extensive computational studies in several laboratories, including ours [13,15]. COMT, an enzyme that catalyzes the methyl transfer from S-adenosyl methionine (SAM) to catecholate, can be used to illustrate the role of the electrostatics interactions in catalysis. As shown in **Figure 4a**, the reaction consists in the transfer of a positively charged methyl cation from SAM to the negatively charged substrate (the catecholate anion). Thus, the reaction proceeds from charged reactants to neutral products. In aqueous solution this process is quite difficult because water molecules stabilize charged species by means of hydrogen bond and ion-dipole interactions. The solvation shell around the ions must be considerably distorted and the ions desolvate to allow the methyl transfer between them. This implies a free energy penalty to reach the TS from the fully solvated and separated reactants, increasing the activation free energy and thus reducing the rate of the reaction (**Figure 3**). In contrast, the active site of the enzyme is almost perfectly preorganized

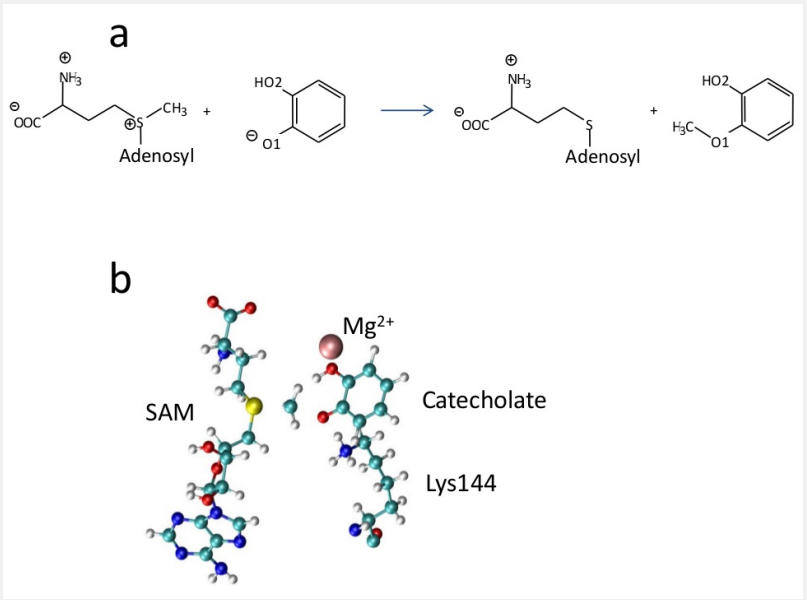

Figure 4. Reaction catalysed by Catechol O-Methyl Transferase. (a) *A methyl transfer from S-Adenosyl-Methionine to Catecholate.* ***(b)*** *Representation of the reaction Transition State in the enzyme with a* Mg^{2+} *ion bounded to the substrate.*

to stabilize the charge distribution of the TS, where the charge transfer between the two reacting fragments is considerably advanced because the methyl group is placed in between the donor sulphur atom and the acceptor oxygen atom (**Figure 4**).

The differences in the behaviour between the solvent and the enzyme can be illustrated following the free energy change associated to the reaction as a function of two coordinates: one measuring the advance of the reaction (the degree of methyl transfer from the donor to the acceptor atom) and the other one measuring the changes in the environment (which can be obtained from the electrostatic potential created by the enzyme on the donor and acceptor atoms). These two coordinates are qualitatively illustrated in **Figure 5a**. The free energy surfaces obtained as a function of these coordinates for the catalyzed and the uncatalyzed reactions are shown in **Figure 5b**. The most probable reaction paths from reactants (R) to products (P) crossing the TS are depicted as discontinuous lines on the free energy surfaces. The free energy surface corresponding to the reaction in aqueous solution shows that, in order to reach the reaction TS from the reactants, a large change must be done, not only in the reaction coordinate (i.e, the methyl group must be positioned between the donor and the acceptor atoms) but also in the environmental coordinate. Solvent molecules placed around the reacting fragment must be reordered to favour the methyl transfer. This large displacement along the environmental coordinate involves a large contribution to the activation free energy. However, the free energy surface corresponding to the same reaction in COMT clearly shows that in order to reach the reaction TS from the reactants (the Michaelis complex) a much smaller change is needed in the environmental coordinate. This illustrates the concept of *preorganization*: the electrostatic properties of the active site already in the Michaelis complex are close to that needed to reach the reaction TS, and thus a much smaller work must be done on the environment. This results in a smaller contribution to the activation free energy and thus in an increase in the observed rate constant with respect to the counterpart reaction in solution. The ultimate reason for this preorganization is found in the protein structure that results from the folding process and substrate binding. In this particular case, the presence of a conserved magnesium ion in the active site (**Figure 4a**) clearly contributes to create the adequate electrostatic environment for the reaction.

Perspectives in the field. Enzyme Design

Computational studies of enzymatic reactivity render a detailed knowledge on the source of the catalytic efficiency of natural enzymes that can be then used to guide the design of new generations of biocatalysts. The advantages of the use of biocatalysts in chemical and biochemical industrial processes are due not only to their ability to speed up chemical reactions by several orders of magnitude, but also with other inherent features of enzymes, such as their chemo-, regio- and stereoselectivity, and the ability to work under mild conditions of temperature and pressure. With the knowledge acquired from computational simulations, modified or even completely new enzymes can be designed and then prepared by means of protein engineering techniques. These new biocatalysts could then be used to catalyse the production of new valuable molecules or to substitute traditional industrial processes by cheaper, more efficient and more environmentally

friendly procedures.

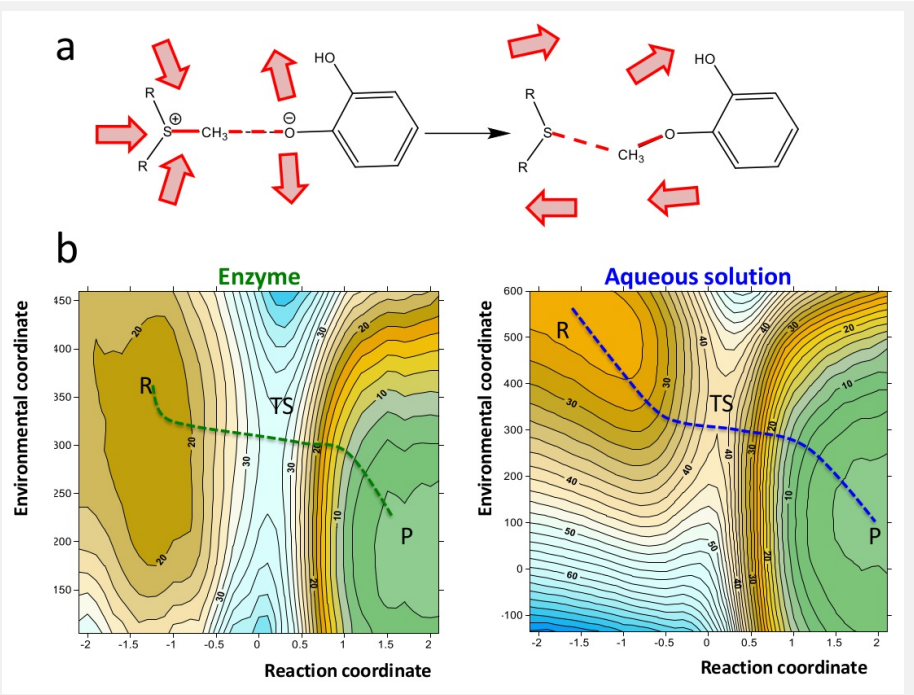

Figure 5. Two-dimensional coordinate description of the O-methyl transfer reaction. (a) *Catechol methylation in COMT and in aqueous solution can be followed using a reaction coordinate describing the degree of methyl transfer (changes in the solid and dashed red lines) and an environmental coordinate describing the changes in the surroundings (reorganization of red arrows).* ***(b)*** *The Free Energy Surfaces for the reaction in COMT and in aqueous solution show the different degree of preorganization in both environments. The reaction needs a larger change along the environmental coordinate in solution than in the enzyme.*

Different protein designs proposed to catalyse new chemical reactions during the past years can be classified, according to the strategy used in their development, into those based on directed evolution, rational design, or a combination of them known as semi-rational design [16]. Directed evolution refers to strategies inspired in natural evolution and consists in obtaining new proteins with new functions after some mutations or by recombination of protein fragments. The advantage of this strategy is that no structural information is needed *a priori* and that distant regions of the sequence space can be explored. Instead, rational design refers to the introduction of direct mutations of selected residues on specific positions of an already existing protein. These mutations, mostly in the active site or its close surroundings, are inspired in the analysis of data obtained from different sources, such as X-ray diffraction or, most recently, from computational simulations. When a protein without the desired catalytic properties is used as a scaffold to support an active site designed from scratch, the process is known as *de novo* design. This kind of rational design, pioneered by Mayo and co-workers [17], is based in the knowledge of the chemical reaction to be catalysed and, in particular, in its TS. In this case the first step for the design process is the construction of a minimalist active site to stabilize the TS charge distribution. This active site, often known as a *theozyme*, [18], is just a cluster of amino acids placed at adequate positions around the substrate to promote the reaction. At this stage, quantum mechanical methods are needed to properly describe the TS at the electronic level. Later, the minimal active site model is placed into an existing protein scaffold, selected from existing structural data bases. This step consists of several cycles of sequence design and protein structure optimization, followed by the ranking or scoring of the designed candidates.

The field has evolved from simply structural to more functional strategies, and from the design of just the primary coordination sphere of the active site to that of the secondary coordination sphere and beyond. Nevertheless, the amount of reactions catalyzed by computational based designed enzymes remains limited and, with some exceptions, the rate enhancements reached by these new biocatalysts is usually far from those of natural enzymes. The development of any of the different protein engineering strategies is hampered by the limitations of an incomplete understanding of enzyme structure–function relationships as well as by the inherent limitations of the employed experimental and computational techniques.

The design of new enzymes can be broadened by introducing metal ions in the active site. Metal-containing enzymes constitute a promising field due to the combination of the best features of homogeneous catalysis with enzyme catalysis; the broad catalytic scope and the high activity and selectivity under mild conditions, respectively. Nevertheless, since metal ions, or metal complexes, are involved in the process, the difficulties of designing this kind of biocatalysts dramatically increases [19].

From the computational point of view, different improvements have been incorporated during the past decade, such as considering the flexibility of the backbone of the protein scaffold, which was originally assumed rigid. As mentioned by Baker [20], *de novo* based design strategies can fail due to an imperfect theozyme, which does not represent the real TS of the reaction, a distortion of the designed active site into a given protein scaffold, or due to the effect of the long range electrostatic interactions and/or protein dynamics that can be incompatible with catalysis. Thus, further improvements on the *de novo* design of new enzymes must be based in a better localization and characterization of the TS by means of higher level computational methods.

The knowledge and simulation of long range electrostatic interactions can be used as a guide to the design of the new enzymes. Mutations far from the active site can be proposed to improve this kind of interactions. These methodologies can be extended to analyze the influence of mutations in steps like substrate binding or product release. This could improve the substrate affinity and/or the catalytic turnover, respectively. Consequently, the use of flexible molecular models treated by QM/MM multiscale methods, where the protein is introduced explicitly in the calculations, can be the bedrock of future successful studies. A proper sampling of the protein conformational landscape, performed by molecular dynamics simulations, could give also information related with the capability of a designed enzyme to catalyze a multistep-process. In this case, the active site of the enzyme has to be prepared not only to stabilize one TS but also to accommodate and stabilize the system for different steps. In addition, we should consider not only the TS stabilization but also the differential stabilization of the TS with respect to the reactants or Michalis complex [21].

The final goal in this field would be the design of a particular amino acid sequence that will fold into a particular structure with a desired function. This is still a chimera, but the incredibly rapid developments achieved in the last years allow predicting milestones truly breath-taking. Reaching this target will complete the round trip between theory and experiment, confirming theories and

computational tools developed in the field of the simulations of enzyme catalysis.

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