FEBS Letters 580 (2006) 2170–2177

Hypothesis

The catalytic power of enzymes: Conformational selection or transition state stabilization?

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Received 18 January 2006; revised 19 March 2006; accepted 20 March 2006

Available online 30 March 2006

Edited by Judit Ovádi

Abstract **The mechanism by which enzymes produce enormous rate enhancements in the reactions they catalyze remains unknown. Two viewpoints, selection of ground state conformations and stabilization of the transition state, are present in the literature in apparent opposition. To provide more insight into current discussion about enzyme efficiency, a two-state model of enzyme catalysis was developed. The model was designed to include both the pre-chemical (ground state conformations) and the chemical (transition state) components of the process for the substrate both in water and in the enzyme. Although the model is of general applicability, the chorismate to prephenate reaction catalyzed by chorismate mutase was chosen for illustrative purposes. The resulting kinetic equations show that the catalytic power of enzymes, quantified as the k_{cat}/k_{uncat} ratio, is the product of two terms: one including the equilibrium constants for the substrate conformational states and the other including the rate constants for the uncatalyzed and catalyzed chemical reactions. The model shows that these components are not mutually exclusive and can be simultaneously present in an enzymic system, being their relative contribution a property of the enzyme. The developed mathematical expressions reveal that the conformational and reaction components of the process perform differently for the translation of molecular efficiency (changes in energy levels) into observed enzymic efficiency (changes in k_{cat}), being, in general, more productive the component involving the transition state.**

Keywords: Enzyme efficiency; Transition state stabilization; Substrate conformational selection; Ground state destabilization; Kinetic models; Chorismate mutase

1. The problem

Enzymes are biological catalysts producing rate enhancements up to 10^{17} fold with respect to uncatalyzed reactions in water [1]. In spite of the vast amount of data in the literature, a complete explanation concerning enzyme efficiency remains open [2–5]. In particular, the question whether the catalytic power of enzymes involves the stabilization of the transition state (TS) or the selection of ground state (GS) conformations is under debate. In this regard, the proposal of Pauling [6] that an enzyme achieves catalysis only by net stabilization of the TS has been a central paradigm in enzymology during years. However, recent computational studies [7,8] on the chorismate to prephenate reaction catalyzed by chorismate mutase (CM) suggested that the rate of the reaction is strongly dependent on the formation of GS conformers that can convert directly to the TS.

In this study, a kinetic model of enzyme catalysis which includes both the conformational (pre-chemical) and the TS (chemical) components will be explored. Our aim was to help to bridge the gap between these apparent opposite views. To this end, our approach focuses on characterizing the translation of these molecular properties into meaningful kinetic expressions to allow a quantitative analysis of their relative contribution to enzyme efficiency. The CM was selected as an example as this enzyme is a key system for the current debate. Nevertheless, the ideas and equations herein presented are intended to be of general applicability.

2. A system example: chorismate mutase

The isomerization of chorismate to prephenate is catalyzed by CM with a rate enhancement (k_{cat}/k_{uncat}) of 1.9×10^6, where k_{cat} and k_{uncat} are the apparent rate constants for the enzymatic reaction and the uncatalyzed reaction in water, respectively [1]. The reaction is a crucial step in the biosynthesis of aromatic amino acids in microorganisms and plants. Chemically speaking, the isomerization is a Claisen rearrangement [9], which proceeds, as demonstrated by Knowles and coworkers [10,11], through a "chair-like" transition state for the atoms of the [3,3]-pericyclic region, both in solution and in the enzyme. This implies that the enolpyruvyl side chain must be positioned over the cyclohexadienyl for the isomerization reaction (see Fig. 1). Isotope effects on the enzymatic and non-enzymatic reactions of CM revealed a highly asymmetric TS in which C–C bond formation is lagging considerably behind C–O bond cleavage [12,13]. Is because of these properties: (i) the reaction involves an intramolecular rearrangement of substrate to product without formation of covalent bonds between the enzyme and the substrate and (ii) the molecular mechanism is the same both in the enzyme and in solution, that the

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Abbreviations: CM, chorismate mutase; GS, ground state; GSD, ground state destabilization; GSS, ground state stabilization; NAC, near attack conformer; NMR, nuclear magnetic resonance; QM/MM, quantum mechanics and molecular mechanics; TS, transition state; TSS, transition state stabilization

0014-5793/$32.00
doi:10.1016/j.febslet.2006.03.060

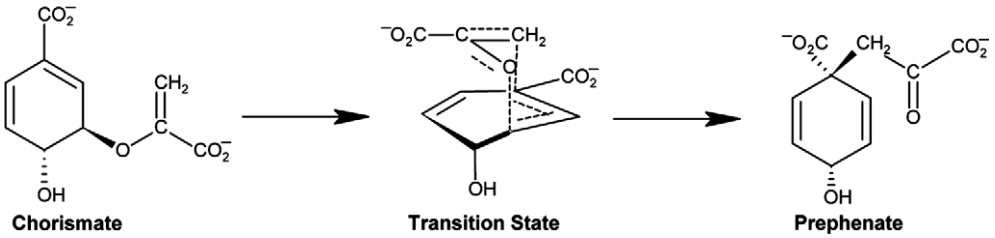


Fig. 1. The chorismate to prephenate isomerization through the proposed "chair-like" transition state where the enolpyruvyl side chain is positioned over the cyclohexadienyl.

isomerization of chorismate to prephenate catalyzed by CM has become central in the study of enzyme efficiency both from experimental and theoretical approaches; a summary follows.

Nuclear magnetic resonance (NMR) studies [14] showed that an equilibrium between two chorismate conformers, pseudodiequatorial and pseudodiaxial, is present in water, being more abundant the pseudodiequatorial (88%) than the pseudodiaxial (12%) form [14]. The bond breaking and making process is presumed to start from the pseudodiaxial conformer, which is capable of progressing to the transition state [15]. Two enzymic pathways can be considered [16]: (i) the enzyme binds selectively the reactive pseudodiaxial conformer or (ii) the enzyme binds the predominant unreactive pseudodiequatorial conformer, which undergoes the conformational change to the pseudodiaxial form in the enzyme.

Based on secondary tritium isotope effects [16], the pathway (ii) mentioned above was eliminated. In addition, a proton transfer from a general acid at the active site was proposed [16]. It is worth mentioning that these experiments were carried out on a bifunctional CM. Kinetic and ^{13}C NMR studies on a monofunctional CM, which lacks the confounding effects due to associated enzyme activities, showed [17] that the kinetic parameters of the monofunctional CM are remarkably insensitive to pH and display no solvent effect. These results allowed the authors [17] to discard that the rate-limiting transition state of the reaction involved a proton transfer and to conclude that there was no reason to suggest that anything other than a simple and rapid pericyclic process occurred at the active site. It was also proposed [17] that CM binds initially the pseudodiaxial conformer (pathway (i), see above). Fourier transform infrared studies [18] were consistent with this hypothesis. It was stated [18] that much, if not all, of the rate acceleration derives from selective binding, with some additional contribution possible from electrostatic stabilization of the TS.

Hilvert and coworkers showed by nuclear Overhauser effect experiments [19] that, although a significant proportion (12%) of chorismate molecules display the pseudodiaxial conformation in water [14], the enolpyruvyl side chain is not positioned over the cyclohexadienyl (a condition needed for the isomerization reaction). The authors suggested [19] that CM could substantially increase the probability of rearrangement by selectively binding the pseudodiaxial form and by correctly orienting the enolpyruvyl side chain.

The role of conformational transitions in CM mechanism has also been tested by theoretical methods. By geometry optimizations in the gas-phase, Karplus and coworkers found [20] two structures in diequatorial conformations ($DIEQ_1$ and $DIEQ_2$) and three structures in diaxial conformations (CHAIR, DIAX and ex-DIAX). CHAIR, which bears the side-chain properly positioned over the ring, is the only active conformation. DIAX, which displays the side-chain over the ring but in an orientation not suitable for reaction, could correspond to the structure determined by Copley and Knowles [14]. Ex-DIAX displays the side-chain in an extended conformation. Finally, both diequatorial conformations are inactive, being the conformation of $DIEQ_2$ more distant to the active CHAIR than the conformation of $DIEQ_1$. In agreement with the experiments of Hilvert group [19], it was observed [20] that the active CHAIR conformer was not stable in solution. Quantum mechanics and molecular mechanics (QM/MM) molecular dynamics simulations in the enzyme of the CHAIR (active), and $DIEQ_1$ and DIAX (both inactive but able to be transformed into CHAIR in the active site) showed that, contrary to what it happened in solution, CHAIR remained stable in the active site whereas, in contrast, $DIEQ_1$ and DIAX were not stable in the active site and were both converted to the CHAIR conformer. It was postulated [20] that the enzyme binds the more abundant nonreactive conformers and it transforms them into the active form previously to the chemical reaction. This proposal is in agreement with the findings [21] by Wolfenden and coworkers from NMR experiments, which suggest that substrates appear to be bound by enzymes, initially, in forms closely related to the most abundant structures in solution.

The importance of GS conformations in determining enzyme efficiency has been studied by Bruice group by making use of the so-called near attack conformers (NACs) [22,23]. NACs were defined as GS conformations in which the reacting atoms are at van der Waals distance and at an angle resembling (±15°) the bond to be formed in the TS. With this definition in mind, NACs could be imagined [8] as the door through which the ground state must pass to become the TS. Within this context, it was found that the population of chorismate present as a NAC conformation is 10^{-4}% in water whereas it consists of 30% in the enzyme. It was concluded [7] that the relative rate of the isomerization of chorismate to prephenate is overwhelming dependent on the efficiency of formation of NACs in the ground state. This conclusion was confirmed in a recent computational study [8] performed by the same laboratory. Remarkably, the authors found [8] that transition state stabilization (TSS) accounts for only 10% of the efficiency of the enzymatic reaction.

The NAC approach has been used by other laboratories with contradictory results [24,25]. The major weakness of NAC hypothesis lies probably in the apparent arbitrariness present in its definition. Thus, the NAC concept has been criticized by some investigators [26,27], who claimed that it cannot be uniquely related to the catalytic effect of the enzyme. Moreover and contrary to the results present above, it was found [27] that the catalytic effect of CM was almost entirely due

to TSS by the electrostatic effect of the active site. It was argued [27] that the apparent NAC effect was not the reason for the catalytic effect but the result of the TSS. Similar outcomes (supporting TSS and disagreeing with a major contribution of NAC effect) have been reported elsewhere [28,29].

The question remains on which are the enzyme–substrate interactions responsible for the catalytic efficiency. Theoretical calculation of interaction energies allowed Szefczyk et al. [30] to identify four arginines (Arg7, Arg63, Arg90, Arg116), one glutamic acid (Glu78), and a crystallographic water molecule as the main components of the electrostatic network responsible for TSS in *Bacillus subtilis* CM. In particular, Arg90 and Arg7 showed [30] the greatest stabilization effects. The hydrogen bonding interaction of Arg90 with the ether oxygen of chorismate has also been identified [29] as the main structural determinant of TSS by CM. These computational results are in agreement with experimental mutagenesis experiments: the arginine/alanine substitution yielded no detectable activity and a 10^6 decrease in k_{cat}/K_m for Arg90Ala [31] and Arg7Ala [32] mutations, respectively. In addition, comparison of CM active site with those from several other species allowed Szefczyk et al. to show [30] that the positions of charged active site residues correspond closely to the optimal catalytic field, indicating that CM has evolved specifically to stabilize the TS relative to the substrate. This result agrees with Warshel's concept of enzyme preorganization [33]. Within this proposal, TSS is basically due to the electrostatic environment provided by the active site of the enzyme, which displays an electric field prepared to accommodate the charge distribution of the TS.

On the other hand and providing more points to the above discussion, the study by Guo et al. [34] suggested that the effect of Arg90 in *B. subtilis* CM catalysis is on stabilizing the reactive substrate conformation (CHAIR) in the active site relative to the solution conformation. The authors argued [34] that their conclusion was not contrary to the TSS hypothesis; yet, they stated that CM uses conformational optimization to lower the TS barrier. In addition, they emphasized [34] that stabilizing the active conformation in the enzyme should not be confused with the proposal by Bruice concerning the role of NACs.

Taken all results together, the question arises as to whether TSS and GS conformational selection are two mutual incompatible concepts. To tackle this issue, Martí et al. [35] proposed an integrated view of enzyme catalysis. By QM/MM methods, these authors found that CM catalytic effect is due to both: (i) a preferential binding of the enzyme to the reactive conformation of the substrate (substrate preorganization) and (ii) a better adaptation of the enzyme to the transition structure of the reaction (enzyme reorganization). It was concluded [35] that "both reorganization and preorganization effects have to be considered as the two faces of the same coin, having a common origin in the effect of the enzyme structure on the energy surface of the substrate". It is worth noting that the enzyme reorganization effect, as defined by Martí et al. [35], is equivalent to the enzyme preorganization effect as defined previously by Warshel [36].

In line with the ideas underlying the approach followed by Martí et al. [35], a kinetic model is presented herein that shows that, in general, both substrate conformational selection (conformational space) and TSS (reaction space) may coexist in enzyme catalysis, being the importance of one space (coin face in Martí et al. [35] words) relative to the other dependent on the relative energies of the structures involved. To avoid the ambiguity that inclusion of NACs can produce, only conformers that correspond to real equilibrium states were used.

3. The conformational/reaction kinetic model

Let us suppose that, for a general reaction, a given substrate presents various inactive conformations, in particular two (S'_1 and S'_2), and one active conformation (S).

For the uncatalyzed reaction in solution we may write:

$$S'_1 \overset{X_1}{\rightleftarrows} S'_2 \overset{X_2}{\rightleftarrows} S \overset{k_2^S}{\rightarrow} P \tag{1}$$

where $X_1 = \frac{[S'_2]}{[S'_1]}$ and $X_2 = \frac{[S]}{[S'_2]}$ are the constants for the substrate conformational equilibria and k_2^S is the rate constant in solution. Eq. (1), although simple, can be suitable for the chorismate to prephenate reaction: S represents the active CHAIR conformation (likely present in a very low concentration, as shown by computational methods [20]) whereas S'_1 and S'_2 represent the inactive pseudodiequatorial and pseudodiaxial conformations, respectively.

The reaction rate in solution is defined as

$$v^S = k_2^S \cdot [S] = k_2^S \cdot \frac{[S_0]}{1 + \frac{1}{X_2} + \frac{1}{X_1 \cdot X_2}} = k_{uncat} \cdot [S_0], \tag{2}$$

where $[S_0] = [S'_1] + [S'_2] + [S]$ is the total substrate concentration, $k_{uncat} = k_2^S \cdot \frac{1}{1+\frac{1}{X_2}+\frac{1}{X_1 \cdot X_2}}$ is the apparent rate constant of the uncatalyzed reaction, and v^S is the initial rate of formation of products. The expression for k_{uncat} can be rearranged to $k_{uncat} = k_2^S \cdot \frac{1}{1+\frac{1}{K_S}}$, where

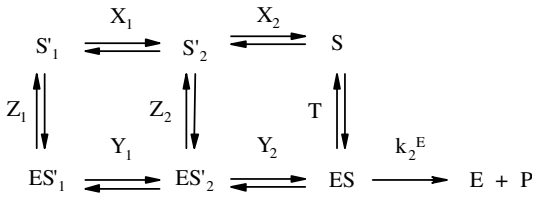
$$K_S = \frac{[S]}{[S'_1] + [S'_2]} = \frac{[\text{substrate active conformation in solution}]}{\sum_i [\text{substrate inactive conformation in solution}_i]}.$$

Thus, k_{uncat} is the product of two terms, one related to the relative energy of the TS and the other to the ratio of concentrations between active and inactive GS conformations. The apparent k_{uncat} tends to the true k_2^S when the concentration of the active conformation is much larger than the sum of the concentrations of inactive conformations.

Eq. (1) and the subsequent expression obtained for k_{uncat} can be useful to discuss solvent effects. The observed overall rate for the chorismate to prephenate rearrangement is 100-fold faster in water than in methanol [14]. There are controversial interpretations for the factors contributing to these solvent effects. Copley and Knowles [14] proposed from NMR studies that 10 out of the 100-fold rate enhancement for chorismate in water was due to the skewed equilibrium; the other 10-fold was attributed to greater stabilization in water for the TS of the rearrangement. Carlson and Jorgensen [15] found from Monte Carlo simulations that the entire 100-fold rate enhancement for the rearrangement in water over methanol could be attributed to the shift to a higher pseudodiaxial population in water. It is worth mentioning that, as pointed out by Karplus and coworkers [20], the structure corresponding to the pseudodiaxial conformation in both studies was not the active CHAIR conformation but the inactive DIAX conformer in the first study [14] and the inactive extended ex-DIAX con-

former in the second [15]. The concentration in water of the CHAIR conformation was found to be [20] very small, much lower than the concentrations corresponding to the structures used in the previous studies. Thus, former predictions [14,15] of solvent effects for the uncatalyzed rearrangement could be in part affected by an inappropriate assignment of the active species. Jorgensen and coworkers realized of the importance of this issue and re-computed their early studies [15] by using Monte Carlo free energy perturbation methods under the NAC framework; in their new results [37], they found a NAC population in water of 82%. This finding contrasts with the value of 10^{-4}% provided by Hur and Bruice [23] and with the conclusion reached by Karplus group [20] for the CHAIR conformation (see above; in principle, one would expect a greater abundance in water for a "true" GS conformation than for a NAC). The conclusion reached by Jorgensen group has been attributed by other authors [7] to the fact that in Jorgensen's NAC definition the necessity for orbital overlap to allow the pericyclic rearrangement was disregarded. Thus, in an MD simulation of chorismate in water, it was found that a NAC population of 50 000 reduced to just one when, in addition to the distance between the atoms forming the new covalent bond, the angles involving the corresponding π-orbitals were considered [7].

For the enzyme catalyzed reaction we may write



(3)

where $X_1 = \frac{[S'_2]}{[S'_1]}$, $X_2 = \frac{[S]}{[S'_2]}$, $Y_1 = \frac{[ES'_2]}{[ES'_1]}$, $Y_2 = \frac{[ES]}{[ES'_2]}$, $Z_i = \frac{[E]\cdot[S'_i]}{[ES'_i]}$ with $i = 1$ or 2, and $T = \frac{[E]\cdot[S]}{[ES]}$ are the constants for the equilibria included in the cycle and k_2^E is the rate constant in the enzyme. The lower branch of Eq. (3) is considered in Ref. [1, p. 107] supposing that the enzyme binds the substrate in the S'_1 conformation and ES'_2 is an intermediate prior to the reactive ES (conformational induction effect). Inclusion here of the upper branch allows for illustrating that conformational induction and conformational selection are equivalent from a macroscopic point of view and for the comparison between k_{cat} and k_{uncat} (see below).

The reaction rate in the enzyme is defined as

$$v^E = k_2^E \cdot [ES] = \frac{[S_0]\cdot[E_0]\cdot k_{cat}}{K_m + [S_0]}, \tag{4}$$

where

$$k_{cat} = k_2^E \cdot \frac{1}{1 + \frac{1}{Y_2} + \frac{1}{Y_1 \cdot Y_2}}, \quad K_m = T \cdot \frac{1 + \frac{1}{K_S}}{1 + \frac{1}{Y_2} + \frac{1}{Y_1 \cdot Y_2}},$$

and $[E_0] = [E] + [ES'_1] + [ES'_2] + [ES]$ is the total enzyme concentration. The expressions for k_{cat} and K_m can be rearranged to

$$k_{cat} = k_2^E \cdot \frac{1}{1 + \frac{1}{K_{ES}}} \quad \text{and} \quad K_m = T \cdot \frac{1 + \frac{1}{K_S}}{1 + \frac{1}{K_{ES}}},$$

where

$$K_{ES} = \frac{[ES]}{[ES'_1] + [ES'_2]} = \frac{[\text{substrate active conformation in the enzyme}]}{\sum_i [\text{substrate inactive conformation in the enzyme}_i]}.$$

Thus, k_{cat} is the product of two terms, one related to the relative energy of the TS and the other to the ratio of concentrations between active and inactive GS conformations of the substrate in the enzyme. The apparent k_{cat} tends to the true k_2^E when the concentration of the active conformation of the substrate in the enzyme is much larger than the sum of concentrations of inactive conformations in the same medium. K_m is the product of two terms, one related to the affinity of the active conformation for the enzyme and the other to both the ratio between active and inactive conformations of the substrate in solution and the ratio between active and inactive conformations of the substrate in the enzyme. The apparent K_m tends to the true T when the concentration of the active conformation of the substrate is much larger than the concentrations of inactive conformations both in solution and in the enzyme.

It is worth mentioning that equations were derived under the steady-state approximation; the concentration of the enzyme was considered negligible compared with that of the substrate and v^E is the initial rate of formation of products so that the substrate has not been appreciable depleted [1].

4. Enzyme efficiency from kinetic equations

The efficiency of the catalyzed relative to the uncatalyzed reaction can be quantified by the ratio of the k_{cat} and k_{uncat} apparent rate constants

$$\frac{k_{cat}}{k_{uncat}} = \left(\frac{k_2^E}{k_2^S}\right) \cdot \left(\frac{K_{ES}}{K_S} \cdot \frac{K_S + 1}{K_{ES} + 1}\right). \tag{5}$$

Eq. (5) shows the relationship between the observed efficiency ratio (k_{cat}/k_{uncat}) and both the rate constants of the chemical reactions (chemical component) and the equilibrium constants for the equilibrium between reactive and unreactive conformations (pre-chemical component) of the substrate both in the enzyme and in solution. Interestingly, the pre-chemical component embraces two terms: the K_{ES}/K_S ratio and a modulating factor which includes K_S (a measure of the relative abundance of the active species in solution) and K_{ES} (a measure of the relative abundance of the active species in the enzyme) apparent equilibrium constants.

It is worth noting that

$$\frac{K_{ES}}{K_S} = \frac{\frac{[\text{substrate active conformation in the enzyme}]}{\sum_i [\text{substrate inactive conformation in the enzyme}_i]}}{\frac{[\text{substrate active conformation in solution}]}{\sum_i [\text{substrate inactive conformation in solution}_i]}}$$

is an index of the capacity of the enzyme to induce the active conformation of the substrate in the substrate-enzyme complexes relative to solution (conformational induction effect). The latter expression can be rearranged as

$$\frac{K_{ES}}{K_S} = \frac{\frac{[\text{substrate active conformation in the enzyme}]}{[\text{substrate active conformation in solution}]}}{\frac{\sum_i [\text{substrate inactive conformation in the enzyme}_i]}{\sum_i [\text{substrate inactive conformation in solution}_i]}},$$

and the $\frac{K_{ES}}{K_S}$ ratio can be taken now as an index of the selectivity of the enzyme towards active and inactive substrate

conformations in solution (conformational selection effect; see Refs. [38,39] for a discussion on the thermodynamic equivalence between conformational induction and selection concepts).

Eq. (5) can be used to compare the relative contributions of the chemical and pre-chemical components to enzyme efficiency. For the first component, a direct relationship was obtained between $\frac{k_2^E}{k_2^S}$ and $\frac{k_{cat}}{k_{uncat}}$. Thus, if k_2^E is n times k_2^S, the same result makes for k_{cat} relative to k_{uncat}. However, for the second component, the translation of conformational efficiency $(\frac{K_{ES}}{K_S})$ into observed enzymic efficiency $(\frac{k_{cat}}{k_{uncat}})$ is more complex.

To examine the conformational contribution to enzyme efficiency, three simulations were performed by varying K_S under three fixed K_{ES}/K_S values (10^6, 10^9, and 10^{12}, see Fig. 2A).

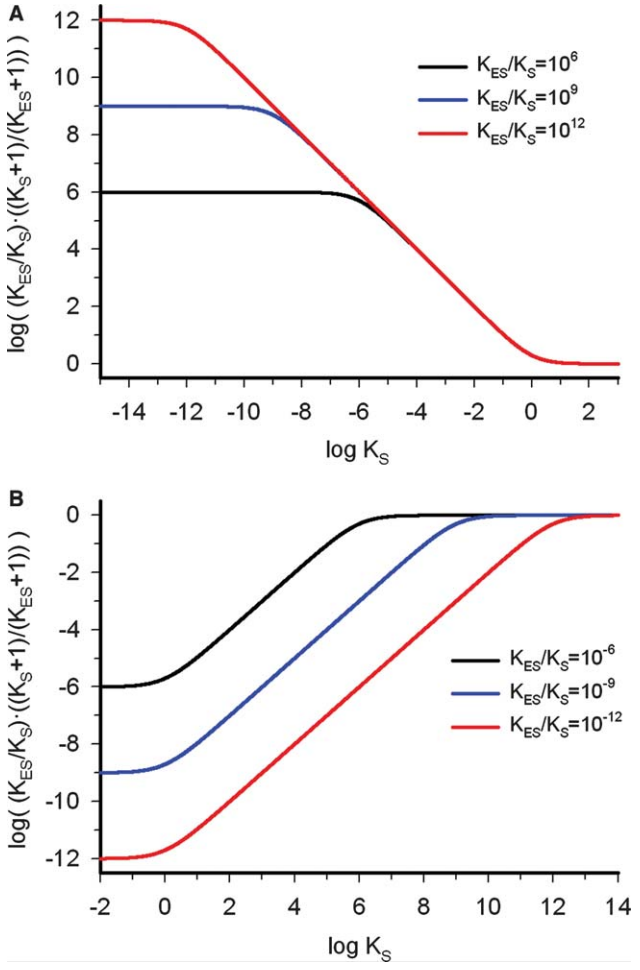


Fig. 2. Simulation of the conformational selection/induction component of enzyme catalysis as a function of substrate conformational equilibrium in solution, both in logarithmic units (see Eq. (5)). (A) In the simulations it is assumed that the substrate active conformation is much more stable in the enzyme than in solution: $K_{ES}/K_S = 10^6$ (black line), $K_{ES}/K_S = 10^9$ (blue line), and $K_{ES}/K_S = 10^{12}$ (red line) are kept constant along the respective curves. Two asymptotes are obtained: the lower one, equal to zero, for $K_S \gg 1$ and the upper one, equal to $\log K_{ES}/K_S$, for $K_S \ll K_S/K_{ES}$. Between them the response is approximately linear with a slope close to -1. (B) In the simulations it is assumed that the substrate active conformation is much less stable in the enzyme than in solution: $K_{ES}/K_S = 10^{-6}$ (black line), $K_{ES}/K_S = 10^{-9}$ (blue line), and $K_{ES}/K_S = 10^{-12}$ (red line) are kept constant along the respective curves. Two asymptotes are obtained: the upper one, equal to zero, for $K_S \gg K_S/K_{ES}$ and the lower one, equal to $\log K_{ES}/K_S$, for $K_S \ll 1$. Between them the response is approximately linear with a slope close to +1.

Note that we are considering only those systems in which the substrate active conformation is much more stabilized in the enzyme than in solution ($K_{ES}/K_S \gg 1$). For each of the curves, three regions can be distinguished, a left-hand upper asymptote approaching $\log K_{ES}/K_S$, a right-hand lower asymptote approaching 0, and an approximately linear function depicting a slope close to -1 in between. This central region spans between $\log (K_S/K_{ES})$ and 0 on the abcisae axis.

The curves may be described as follows. Lower asymptote ($K_S \gg 1$): The contribution of the conformational component is negligible, $\frac{k_{cat}}{k_{uncat}} \approx \frac{k_2^E}{k_2^S}$. Upper asymptote ($K_S \ll K_S/K_{ES}$, and, consequently, K_S and K_{ES} are both $\ll 1$): The importance of the conformational selection/induction component increases as K_S decreases, with a limiting value equal to the K_{ES}/K_S ratio. Within this region, a factor of n in K_{ES} relative to K_S will produce an increase in the same quantity in k_{cat} relative to k_{uncat}. However, in absolute terms, the conformational efficiency of the enzyme would be small as K_{ES} is much lower than one. Central linear region ($K_S/K_{ES} < K_S < 1$): A value of n for the $\frac{K_{ES}}{K_S}$ ratio will produce a value lower than n for the $\frac{k_{cat}}{k_{uncat}}$ ratio. This suggests that, in general, the enzyme gains more efficiency by acting on the chemical than on the pre-chemical component of the catalytic process. Nevertheless, both components are not mutually exclusive and can be simultaneously present in an enzymic system.

To illustrate the connection between conformational and chemical factors within our model, we will consider three numerical combinations compatible with the CM experimental $\frac{k_{cat}}{k_{uncat}} = 10^6$ value. (i) $K_{ES}/K_S = 10^6$ and $K_S \gg 1$: in this case, the resulting value for the conformational term of Eq. (5), $\frac{K_{ES}}{K_S} \cdot \frac{K_S+1}{K_{ES}+1} \approx 1$, would make irrelevant the contribution of the pre-chemical component to enzyme efficiency; this condition is consistent with the findings [37] by Jorgensen group. (ii) $K_{ES}/K_S = 10^6$ and $K_S \ll 10^{-6}$: in this case, the resulting value (10^6) for the conformational term suggests that, for a virtual enzyme with observed kinetic parameters similar to CM, it would not be necessary an increment in the rate constant for the chemical reaction (k_2) to achieve the experimental k_{cat}/k_{uncat} ratio. (iii) $K_{ES}/K_S = 10^6$ and $K_S = 10^{-3}$: in this case, the conformational component amounts 10^3, approximately; then, to achieve $k_{cat}/k_{uncat} = 10^6$, the contribution of the TS component would match the conformational one. The same result (equivalence between conformational and TS contributions) would be obtained for the two other curves ($K_{ES}/K_S = 10^9$ and $K_{ES}/K_S = 10^{12}$) by assuming $K_S = 10^{-3}$; moreover, it could be also obtained for an additional $K_{ES}/K_S = 10^3$ curve within the upper asymptote ($K_S \ll 10^{-3}$). These results are in good agreement with those from a recent study [40]. Multiple high-level QM/MM reaction pathways in CM provided [40] a calculated average TSS of 46.2% of the experimentally observed catalytic effect, and thereby the remaining 53,8% was attributed to conformational effects.

In terms or the proposed model, it is interesting to examine the impact of the destabilization of the active form of substrate (in *ES* complex) on k_{cat}/k_{uncat}. In correspondence with the analysis above, three simulations were performed by varying K_S under three fixed K_{ES}/K_S values (10^{-6}, 10^{-9}, and 10^{-12}, see Fig. 2B). We see that in each of the curves, the conformational factor contributes negatively to the observed kinetic ratio, being its effect greater as K_S decreases, and with a limiting value equal to the predetermined K_{ES}/K_S. In principle, it seems

that destabilization of substrate active form does not seem a right strategy for the enzyme unless this choice would lead to a decrease in the energy barrier of the chemical step. To better understand these interrelated relationships, an energetic approach may be helpful.

4.1. Water and enzyme: two energetic landscapes for the reaction process

To discuss the problem from an energetic point of view, Fig. 3 depicts a diagram showing the pre-chemical and chemical spaces of the process both in water and in the enzyme. In this figure, catalytic efficiency is proposed to be obtained either by lowering the barrier of the transition state (the affinity of the enzyme for the substrate transition state is increased: TSS) or by increasing the energy of the inactive conformations (ground state destabilization (GSD) of the inactive conformations). Our results have shown that although both alternatives are compatible, it seems more favorable to the enzyme to opt for TSS. It should be noted however that, in our modeling approach, TSS and GSD have been taken as two autonomous events. As a consequence, the resulting expression for k_{cat} could be arranged as the product of two independent terms, one concerning the pre-chemical and the other the chemical space. Yet, it seems clear that there must exist a much stronger structural similarity between the TS and the active species than between the TS and any of the inactive species [29,35]. Thus, if an enzyme managed to lower the energetic cost of a reaction by acting on the substrate TS, indirectly it would be acting also on the substrate conformational landscape [25], increasing the energy of the substrate inactive conformations relative to the active one. It may be then hypothesized that GSD is a consequence of TSS. This proposal is in line with previous work by Warshel group where it was stated [27] that the apparent NAC effect was not the reason for the catalytic effect but the result of the TSS. It is also in agreement with a study of Karplus and coworkers where the stabilization of the substrate active conformation (CHAIR) in the enzyme relative to solution was explained [34] by arguing that CM uses conformational optimization to lower the TS barrier.

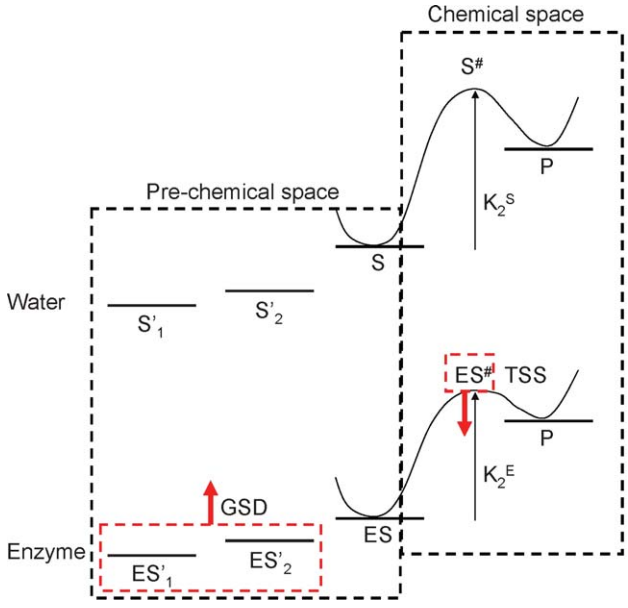


Fig. 3. Energy diagram of the pre-chemical and the chemical components of a reaction both in water and in the enzyme. Enzyme efficiency is obtained either by lowering the barrier of the transition state (transition state stabilization: TSS) or by increasing the energy of the inactive conformations (ground state destabilization of the inactive conformations: GSD). Macroscopically speaking, GSD of the inactive conformations is equivalent to say that the enzyme binds selectively the active conformation (substrate conformational selection) or the enzyme binds the predominant inactive conformation, which undergoes the conformational change to the active form in the enzyme more easily than in solution (substrate conformational induction).

The likely connection between TSS and GSD effects adds an extra difficulty to the correct interpretation of enzyme function. A detailed discussion about this issue, namely, the stabilization of one state will likely affect the stability of neighboring states within the free energy profile of a given reaction, can be found elsewhere [5]. As it was pointed out [5], the highly cooperative nature of enzyme mechanism renders impossible an absolute partitioning of catalytic contributions into independent components. In this study [5], numerous examples were shown in which the energetic and functional interconnections of binding and catalysis were present, and the authors emphasized the impossibility of separating the binding and catalytic contributions on a residue-by-residue basis. This coupling between binding and catalysis has been observed also in CM, where one residue (Arg90) has been found to incorporate both effects: catalytic (TSS) [29,30] and binding (stabilization of substrate active conformation) [34]. As indicated above, the model herein presented treats the chemical and pre-chemical steps as independent events. However, since the extension of coupling between binding and catalysis varies from one residue to another and it also depends on the particular enzyme considered, it seems difficult to formulate a quantitative generalization of this concept in a kinetic model.

We would like to remark that the definition we have used for GSD is not exactly the same effect as the substrate destabilization discussed by others. GSD is usually defined as the lowering of energy barrier due to the increased energy of the enzyme-bound substrate comparing to the unbound form [3,27,30]. This effect is equivalent to an increase of the value of the T equilibrium constant as defined in Eq. (3), an outcome that would lead to a decrease of the barrier for the chemical reaction if it would serve for pushing ES towards $ES^{\#}$ both in energy and structure. However, as it was shown in Fig. 2B, increasing the energy of the active form of substrate in ES complex (GSD of the active conformation) can have counter-productive effects since it can produce a correlated energetic stabilization of the inactive forms of substrate in ES′ complexes (ground state stabilization (GSS) of inactive conformations), and, hence, to a hampering of the catalytic process.

For illustrative purposes, and according to the arguments found in this work, the evolutionary transformation of free energy reaction profiles might be imagined to have appeared in two steps (see Ref. [5] for a detailed analysis of enzyme mechanism). First, uniform binding: the energy profile of the substrate free in solution suffers a constant shift that does not alter the relative energy between levels. Then, differential binding occurs. This step could have been taken under the following strategies: (i) TSS: the enzyme environment adapts its shape to favor the TS (by electrostatic or other attractive interactions) or (ii) GSD of the active form: the enzyme environment adapts its shape to disfavor the active form of the substrate by some kind of strain. Strategy (i) may involve a

productive uneven displacement of the ground states: since the structure of the substrate active form is more similar to the TS than the inactive forms [29,35], the latter conformations are destabilized relative to the active form (GSD of inactive conformations). The two components of strategy (i), namely TSS and GSD of inactive conformations, correspond to the two coin sides of above-mentioned work by Martí et al. [35], that is, enzyme reorganization and substrate preorganization, respectively. The link between these properties was attributed by these authors to the protein structure, which being preferably adapted to the TS it shows a low enzyme deformation when passing from the substrate active form to the TS structure [35]. Importantly, strategy (ii) should include the necessary condition that the chemical reaction should not be disabled, and to this end the energy increase of the substrate in the ES complex should encompass a structural resemblance to the TS. In addition, to avoid conformational inefficiency (see Fig. 2B), the energies of the substrate inactive conformations should be increased in the same or greater amount than the active form.

A key distinction can be established between above mentioned enzyme strategies. Stabilization (strategy (i)) is precisely defined in terms of complementary functional groups; however, destabilization (strategy (ii)) is not. In other words, since the TS has a well-defined structure, the target for strategy (i) is univocally defined, whereas there can be multiple structural ways to achieve destabilization for strategy (ii), being, probably, a number of them non-productive. Thinking in terms of structural optimization process, enzyme mutation following strategy (i) seems more successful.

It is interesting to consider the energy crossing between solution and enzyme landscapes for a given substrate molecule (Fig. 3). In our hypothesis, we could visualize the enzyme as a microscopic vortex in which the substrate, after entering from solution, probably in its most populated (inactive) form, experiments, first, a driving force towards the active form (destabilization of the inactive conformation relative to the active form) and, subsequently, a driving force towards the transition state (stabilization of the TS relative to the active form). These two driving forces may be linked to the intrinsic flexibility of enzymes, which should not be ignored either [41–44]. The mobility of the enzyme between, in the simplest model, two conformations, one (open) associated to the substrate GSs and the other (closed) associated to the substrate TS, can be crucial in the catalytic process. Furthermore, it can have important implications for drug discovery [39], both for orthosteric and allosteric inhibitor design. Our study focused on the effects of multiple ligand conformations in enzymatic catalysis. Accordingly, protein flexibility (E_{open} and E_{closed} species) was not required. Nevertheless, protein plasticity is implicitly present in our model if we suppose that the conformations of the protein in the ES'_1, ES'_2, ES, and $ES^{\#}$ complexes are not necessarily the same.

5. Concluding remarks

Analysis of enzyme catalysis by combining the conformational and the TS spaces in a single kinetic model allowed the quantitative evaluation of their relative contribution to enzyme efficiency. We found that while the translation of microscopic efficiency (changes in energy levels) into observed macroscopic efficiency (the apparent k_{cat}/k_{uncat} ratio) depends directly on the TS element (the k_2^E/k_2^S ratio), the contribution of the conformational component follows a more complex function, which includes, in addition of the K_{ES}/K_S ratio, the stabilities of the substrate active state both in solution and in the enzyme. Remarkably, the importance that a differential conformational landscape in the enzyme relative to solution can have on catalysis increases as lower is the stability of the reactive conformation in solution.

Our modeling showed that CM, chosen in this work as a system example, seems to gain more efficiency by adapting its structure to the stabilization of the TS rather than to the GS conformations: In Eq. (5), a value of $n > 1$ for $\frac{k_2^E}{k_2^S}$ has a direct effect (the same n value) in $\frac{k_{cat}}{k_{uncat}}$; in contrast, a value of $n > 1$ for $\frac{K_{ES}}{K_S}$ results in a value of $n(\frac{1+\frac{1}{K_S}}{n+\frac{1}{K_S}})$, which is lower than n, for the conformational factor, and accordingly for the observed kinetic ratio. Yet, this result should not be taken as a universal property, as many enzymes are governed by mechanisms other than that of CM.

Equations were developed aiming at bridging the gap between the two main approaches to the study of enzyme efficiency. Our intention was both to help to conciliate a number of controversial concepts and to provide a framework to more focused debate.

Acknowledgments: We thank Iñaki Tuñón for critical reading of the manuscript and helpful comments. This work was supported in part by Grant SAF2004-06134 from Dirección General de Investigación, Ministerio de Educación y Ciencia (Spain).

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