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Electrostatic Basis for Enzyme Catalysis

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Contents

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1. Introduction

Enzymatic reactions play a fundamentally important role in controlling and performing most life processes.[1−3] Thus, understanding how enzymes work has both fundamental and practical importance. In this respect it is crucial to understand what is the origin of the enormous catalytic power of enzymes, which remains one of the challenges of modern biophysics. Although many elements of this puzzle were elucidated by biochemical and structural studies, the source of the catalytic power of enzymes has not been widely understood and, clearly, has not been agreed upon by the scientific community (e.g., see ref 4). The current consensus is sometimes reduced to statements such as, "the enzyme binds the transition state stronger than the ground state" or "the catalytic groups are perfectly oriented". However, such statements are not sufficient to explain this catalytic power since the real question is how this differential binding is accomplished and what are the actual catalytic groups.

The issue of the origin of enzyme catalysis is, in fact, sometimes confused and trivialized by attributing it to the selection of the reference state (see below) and implying that the enormous acceleration by 10 orders of magnitude is well understood since binding energies of ligands by proteins can reach 15 kcal/mol.[5] As will be discussed in this review, the issue is not the binding energy itself but rather the change in binding energy on moving from the reactant state to the transition state. Unfortunately, most attempts to account for the catalytic power of enzymes cannot rationalize binding energies of more than a few kilocalories per mole. The problem becomes more challenging after realizing that some enzymes catalyze their reactions by more than 20 orders of magnitude and that this catalytic effect is entirely due to the active site environment and has very little to do with covalent arguments of the type promoted in refs 5 and 6.

Earlier attempts to quantify the contributions to enzyme catalysis were reviewed in, e.g., refs 1 and 7−11. However, this review will explore the origin of the catalytic power of enzymes in a somewhat more systematic way. It will start by clarifying recent confusions regarding the reference state by introducing a catalytic scale that does not include the well-understood effect of having different mechanisms in the enzyme and in solution as well as the effect of the binding of the reactant state. This will allow us to focus on the effect of the enzyme environment, which must represent the true catalytic effect (see below). We will demonstrate that the effect of the enzyme environment can be much larger than the estimated 15 kcal/mol provided in ref 5. Furthermore, we will point out that a correct rationalization of "even" this 15 kcal/mol effect is an enormous challenge. We will then

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Arieh Warshel is a professor of Chemistry and Biochemistry at the University of Southern California. He was born in Kibutz Sdhe Nahum, Israel, and received his B.Sc. in 1966 from the Technion Institute in Israel. He then earned his M.Sc. in 1967 and his Ph.D. in 1969 from the Weizmann Institute. His Ph.D. with Shneior Lifson and collaboration with Mike Levitt is the basis of many current molecular simulation programs. Between 1970 and 1976, he did postdoctoral research at Harvard University, returned to the Weizmann Institute, and was an EMBO fellow at the MRC in Cambridge. In 1976, he joined the Department of Chemistry at the University of Southern California. He has pioneered computer-modeling approaches for studies of protein functions. These include the development of consistent treatments of electrostatic energies in proteins, the development of hybrid quantum mechanical/molecular mechanics (QM/MM) approaches for studies of enzymatic reactions, the introduction of molecular dynamics simulations to studies of biological processes, and the introduction of microscopic evaluation of thermodynamics cycles in biological systems. Warshel's group focuses on simulations of the structure–function correlation of biological systems. Prof. Warshel is the author of *Computer Modeling of Chemical Reactions In Enzymes and Solutions*, published in 1991. He received the 1993 award of the international Society of Quantum Biology and Pharmacology, and he is a fellow of the Biophysical Society and a recipient of the 2003 Tolman Award. Dr. Warshel enjoys watching meaningless television programs.

Pankaz Kumar Sharma hails from Assam, a distant Indian state on the banks of the Brahmaputra river. He obtained his B.Sc. (with a major in Chemistry) from Cotton College, Guwahati, then moved to Delhi, where he studied Physical Chemistry, and has remained a *nomad*—both physically and "chemically"—ever since. After completing his Masters from Delhi University (1995), he traveled halfway across India to pursue graduate studies in Chemistry at the University of Hyderabad, where he earned a Ph.D. (2002), working with Prof. E. D. Jemmis on (Computational) Organometallic Chemistry. His next stint was as a postdoctoral researcher in Computational Bioinorganic Chemistry with Prof. Sason Shaik at the Hebrew University of Jerusalem, Israel (June 2001 to December 2003). His most recent move has been to the University of Southern California, Los Angeles, where he has been employed, since December 2003, as a postdoctoral researcher in the Theoretical Biophysical Chemistry group of Prof. Arieh Warshel. Essentially a student of science all along, Pankaz's present research interest primarily concerns theoretical studies of enzymatic reactions. His passions include literature, World cinema and music, translating, blogging, writing z-matrixes, and computing transition states.

Mitsunori Kato was born in Aichi, Japan. He received his B.S. degree in 1995 and his M.S. degree in 1998 in Pharmaceutical Sciences from the University of Tokyo. Then he worked for Chugai Pharmaceutical Co., Ltd., in Shizuoka, Japan, from 1998 to 2001. He obtained his Ph.D. degree in Chemistry from the University of Southern California in 2006. His research focuses on improving free energy calculation methods, especially for protein–ligand binding.

review consistent calculations that can reproduce the observed activation energy and the corresponding catalytic effect, and we will point out that such calculations identify electrostatic effects as the key catalytic contribution.

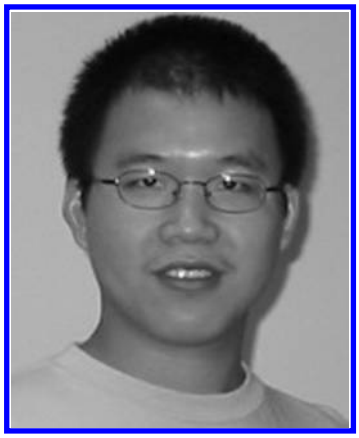
The finding of a major electrostatic contribution to catalysis will be analyzed and shown to reflect the preorganized polar environment of the enzyme active site. The relation of this finding to the local instability in enzyme active sites will also be analyzed.

To establish the unique importance of the electrostatic effect, it is essential to examine the magnitude of other catalytic factors and to show that the contributions are small. This will be done by considering nonelectrostatic proposals and demonstrating that the corresponding effects are small or that the given proposal is inconsistent and poorly defined.

Finally, after clarifying our energy-based considerations, we will conclude with a short overview on the issue of enzyme catalysis and on the prospect for growing consensus in the field.

2. Formulating the Problem and Illustrating the Nature of Different Reference States

Any discussion of catalytic effects requires one to define a proper reference state. In the case of enzymatic reactions, the most natural reference state is the uncatalyzed reaction in solution. However, even with this selection we may have several ways of defining the catalytic effects. For example, we may start with the scale introduced in the pioneering work of Wolfenden and co-workers.[12] This useful scale has established the catalytic power associated with the binding free energy in the enzyme relative to the energy of the transition state in the uncatalyzed reaction in water, but it has still left the field open for possible misunderstandings about the challenge in rationalizing enzyme catalysis. One potential problem is associated with the fact that the mechanisms in enzyme and in solution can be different and this difference is a part of Wolfenden's scale. Another problem arises from the fact that the real challenge in rationalizing enzyme catalysis has not been emphasized by Wolfenden's scale, since it includes the binding free energy of the substrate



Yun Xiang was born in 1978 in Hubei, China. After receiving his B.S. in Chemistry from Peking (Beijing) University in 1999, he came to the United States for graduate study. In 2004, he obtained his Ph.D. in Theoretical Chemistry from New York University under the supervision of Prof. John Zhang. He worked on quantum dynamics and quantum-classical dynamics simulation of polyatomic reactions on metal surfaces, and he helped develop novel methods for quantum calculation of biomolecular energies and structure optimization. Currently he is a postdoctoral research associate at the University of Southern California working with Prof. Arieh Warshel. His research interests focus on computer simulations of protein–ligand binding, protein structure–function correlation, and enzymatic reactions in biological systems.

Hanbin Liu received her B.S. degree (1996) from Tianjin University and her M.S. degree (1999) from Tsinghua University, China. Her masters research advisor was Dezhong Shen. She moved to the U.S. and obtained her Ph.D. from the University of Pittsburgh in 2005 under the supervision of Prof. Kenneth D. Jordan with a thesis on improved sampling in Monte Carlo simulations of small clusters. She is currently working as a postdoctoral research associate with Prof. Arieh Warshel at the University of Southern California. Her current research interests include simulating enzyme catalysis and the isotopic effect.

(whose nature is well understood), whereas the real problem is associated with rationalizing the large change in free energy upon going from the ES to the ES‡ states (i.e. the free energy associated with the k_{cat} and the corresponding $\Delta g^{\ddagger}_{cat}$ of Figure 1).

The comparison of $\Delta g^{\ddagger}_{cat}$ and $\Delta g^{\ddagger}_{w}$ should, of course, reflect the fact that many enzymatic reactions involve mechanisms different from the corresponding solution reactions (e.g., refs 1 and 13−15). However, this effect is well understood and can easily be determined by using a proper thermodynamic cycle without any consideration of the nature of the enzyme environment (see below). Thus, the real puzzle is why the enzyme reaction with the specific chemical groups (e.g. acids and bases) is so much faster than the reaction with the same groups in solution.

Mats Olsson was born in Malmo (Sweden) and received his M.Sc. and Ph.D. in Theoretical Chemistry at Lund University working with B. O. Roos and U. Ryde. In 2000, he joined Dr. Arieh Warshel's research group for postdoctoral studies, where he is currently working as a research associate. His current research interests are focused on modeling electron and proton transfer.

Before discussing the actual evaluation of $\Delta g^{\ddagger}_{w}$, it is important to address some recent misunderstandings about the nature of the reference state. For example, a recent work[5] has implied that the above-mentioned difference, between the regular reaction in water and the water reaction that follows the enzyme mechanism, constitutes a major catalytic effect, that can be considered as a new paradigm in studies

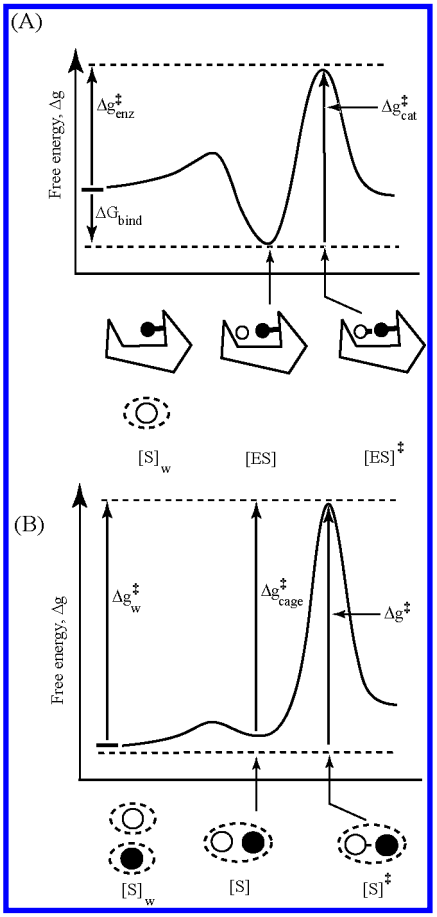


Figure 1. Schematic description of the free energy profile for an enzymatic reaction and that for the corresponding solution reaction. The figure describes the free energies $\Delta g^{\ddagger}_{p}$ and $\Delta g^{\ddagger}_{cat}$ associated respectively with k_{cat}/K_M and k_{cat}. Part B describes the energetics of a reference solution reaction (see also Figure 2).

of enzyme catalysis. Furthermore, it was argued that the difference in mechanism, between the reaction in the enzyme and that in solution, could be described as a covalent catalysis. However, as was pointed out above, the corresponding energetics has been understood quantitatively for a long time (e.g., refs 13−15) and was never a part of the catalytic puzzle. It is also important to distinguish between (i) the effect of having different mechanisms in solution and in the enzyme and (ii) the proposal of real covalent catalysis (where nonchemical groups "bind" to the transition state). At any rate, it is quite clear that studies of the catalytic power of enzymes should focus on elucidating the origin of the large effect of the active site environment rather than on the obvious fact that different mechanisms have different energies in water. Of course, one may ask whether the enzyme environment provides covalent or noncovalent interactions, but this should not be confused with the availability of different mechanisms in the enzyme and in solution. This section will address the above problems and introduce a catalytic scale that does not include the well-understood effect of having different mechanisms in the enzyme and in solution as well as the effect of the reactant-state binding.

To clarify our consideration, it is useful to start with the diagram of Figure 1A, where the activation barrier, $\Delta g^{\ddagger}_{enz}$, corresponds to the overall enzyme proficiency, k_{cat}/K_M (more precisely, it corresponds to k_{cat}/K_D), and $\Delta g^{\ddagger}_{cat}$ corresponds to k_{cat} (or, more precisely, to the enzyme rate constant for the rate-determining chemical step). The energetics of the reaction in the enzyme can now be compared to the corresponding energetics of the reaction in solution (Figure 1B). In this respect, it is important to reclarify the common misunderstanding (e.g. see discussion in ref 16) that "the comparison of a first-order rate constant (in units of s^{-1}) with its second-order counterpart (in units of M^{-1} s^{-1}) is impossible". It is, in fact, rather simple to deal with the issue by considering the free energy profile or the potential of mean force (PMF), for the reaction in water and in the protein, and then dividing the second-order process into the free energy of bringing the fragments to the same cage and the activation barrier of the first-order reactive event. This can be done in an entirely rigorous way (see the cage concept in, e.g., refs 17 and 18). The problem seems to stem from the tendency to talk about concentrations effects and about the ill-defined concept of effective concentration rather than about free energy profiles that are valid for any concentration ranges. At any rate, the comparison of the enzyme and solution reactions can be done either by comparing $\Delta g^{\ddagger}_{cat}$ to $\Delta g^{\ddagger}_{w}$ or by comparing $\Delta g^{\ddagger}_{cat}$ to $\Delta g^{\ddagger}_{cage}$, which corresponds to the case where the reactants are at the same solvent cage (the relationship between $\Delta g^{\ddagger}_{w}$ and $\Delta g^{\ddagger}_{cage}$ corresponds roughly to the 55 M concentration of water and is defined rigorously elsewhere[18,19]).

At this point it might be useful to clarify some points about the transition-state stabilization (TSS) and the reactant-state destabilization (RSD) proposals. The TSS proposal requires that the transition state (TS) in the enzyme will have a lower free energy than the corresponding TS in solution, where the reference energy is taken as the E + S system. The RSD proposal requires that the reactive part of the substrate in the RS of the enzyme will have higher free energy than the corresponding reactive part in the solvent cage (see ref 90). This clear definition has been overlooked in recent works (e.g., refs 84 and 91) that considered the insightful work of Schowen[245] as an example of an early TSS proposal.

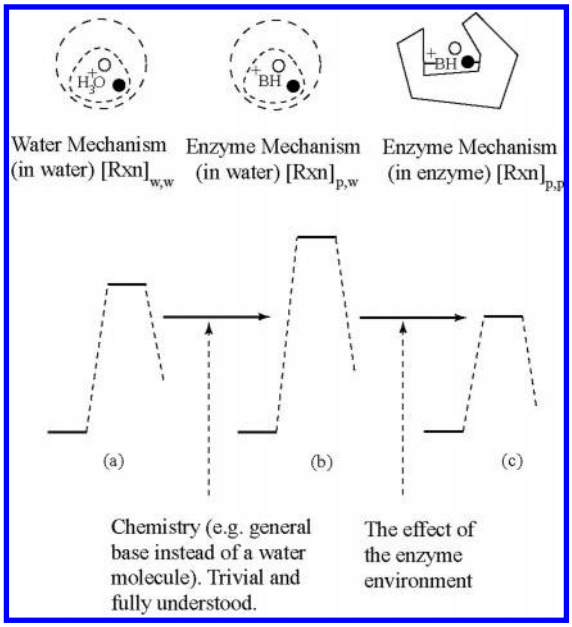


Figure 2. Demonstration of the difference between the activation barriers for (a) a regular reaction in water, (b) a reaction that involves the same mechanism as that in the corresponding enzymatic reaction, and (c) the reaction in the enzyme active site. These three cases correspond to $[Rxn]_{w,w}$, $[Rxn]_{p,w}$, and $[Rxn]_{p,p}$. The upper part of the figure describes schematically the transition states of the different cases for a reaction with and without a general base.

Actually, ref 245 took Jencks's RSD proposal and argued that it can be converted to a TSS proposal by changing the effect of the substrate concentration on the free energy profile. However, the RSD proposal is defined for a standard state concentration (see discussion in section 5.1), and it reflects a unique situation in terms of the corresponding PMF for one substrate molecule and one enzyme molecule. As is clear from Jencks's works, the RSD proposal always meant that the reactive part of the substrate would have a positive binding free energy at the standard 1 M concentration. The RSD proposal cannot be converted to the TSS proposal by a consistent treatment that would consider the same concentration effect on both the reference reaction and the enzyme reaction. In other words, figures of the type presented in Figure 1a and 1b in ref 245 represent two very different proposals and both should be formulated in standard state concentrations. The possible confusion is removed when one actually draws the relevant PMFs for each proposal and evaluates the binding free energies of the RS and TS in the protein and solution reactions. Thus, it is important to realize (despite frequent implications (e.g., ref 84) that all agree that TSS is the way by which enzyme catalysis is accomplished) that almost all early explicit proposals represented RSD proposals. In fact, many of the examples brought in ref 84 represent clear RSD proposals. At any rate, our electrostatic stabilization proposal represents one of the very few non-RSD proposals.

As stated above, it is important to have a reasonable estimate of the energetics of the solution reactions. Fortunately, in many cases it has been possible to estimate the energetics of solution reactions by simple thermodynamic cycles connecting the energetics of different steps (e.g., see refs 1 and 20). Furthermore, the experimental efforts of Wolfenden and co-workers have provided direct estimates for some key reactions.[21] Finally, combining the advance of well-calibrated quantum mechanical calculations of chemical

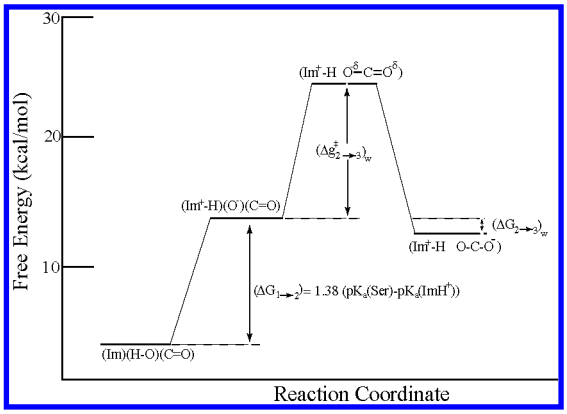


Figure 3. Schematic energy diagram for an amide hydrolysis reaction that occurs in water but follows the mechanism of serine protease (see ref 1 and the text for more discussion). The figure demonstrates that the energetics of the stepwise path can be easily determined from simple experimental information. The energetics of the more complex concerted path can be quantified by ab initio calculations. (Reprinted with permission from ref 25. Copyright 2000 American Chemical Society.)

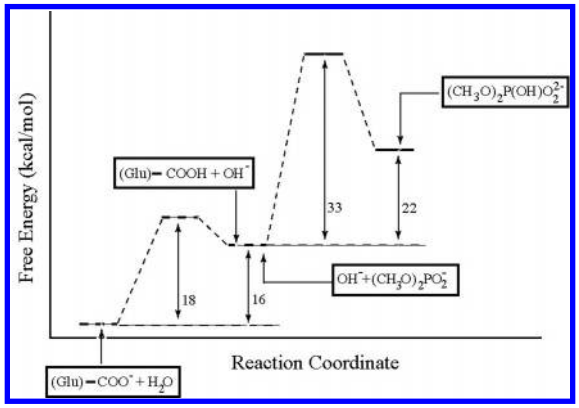


Figure 4. Experimentally determined energy diagram for a reaction that follows the mechanism of SNase but occurs in solution. (Reprinted with permission from ref 111. Copyright 1989 American Chemical Society.)

reactions in solution[22−26] and experimental constraints (e.g., refs 24−26) allows one to estimate quite accurately the energetics of arbitrary reactions in solutions.[27]

Now, fortunately, the difference between the energetics of the actual reaction in solution $[Rxn]_{w,w}$ and those of the reaction that involves the same mechanism as that in the enzyme active site but occurs in water (this latter reference state will provide a "mechanism-filtered" reference state and is designated here by $[Rxn]_{p,w}$) can frequently be assessed almost quantitatively without any quantum mechanical calculations but simply by using pK_a's and related considerations (see below). The relationship between the different reference states is illustrated schematically in Figure 2 for a case when the enzyme reaction involves a general base (B). More specific and quantitative illustrations are given in Figures 3 and 4 in the specific analysis of the reference reactions to the reaction of serine proteases and the reaction of staphylococcus nuclease. As can be seen from both Figures 3 and 4, we can have a significant effect from the involvement of general bases. However, as clarified above, the effect of moving *in water* from one mechanism to another was never the real problem. That is, by considering Figure 3 (see also refs 1 and 28), one realizes that the energetics of the histidine-assisted catalysis in water can be estimated by taking into account two steps. First, a proton transfer from serine to histidine (imidazole), whose energy is approximately given by

$$\Delta G_{1\rightarrow 2} \approx 2.3RT(\mathrm{p}K_a(\mathrm{Ser}) - \mathrm{p}K_a(\mathrm{ImH}^+)) \qquad (1)$$

The second step is an OH^- attack on the carbonyl of the peptide bond, whose energy is known from reactions at high pH (high OH^- concentration).

Obviously, if the reaction is concerted rather than stepwise, it may have a lower barrier. Fortunately, however, the use of ab initio calculations allows us to estimate quite accurately the energy of the reference solution reactions. Such studies[25] indicated that the water-assisted mechanism is concerted and the histidine-assisted mechanism is a more or less stepwise reaction. At any rate, a process that can be assessed by simple pK_a considerations cannot be considered as the central issue in enzyme catalysis. The key issue is the actual effect of the enzyme environment, which is far from trivial.

It might be useful at this point to expand the discussion on the validity of our estimates of the energy of the reference reaction, and to put this in the context of general studies of enzyme catalysis. That is, even the simple considerations used in our early estimates of the energetics of the reference reaction have been quite effective, despite some early criticisms (e.g. ref 29). More specifically, it seems that the general difficulties in estimating activation free energies (and perhaps the assumption that transition-state theory is not fully valid) led most workers, who studied reactions in solution, to focus on rate constants rather than on the determination of the rate-limiting activation barriers. A notable exception is the pioneering work of Guthrie (e.g. refs 30 and 31), who was, however, partially overlooked in physical organic chemistry studies of reaction in solutions. Our realization that only energy consideration can lead to a well defined formulation of enzyme catalysis led us to insist on the evaluation of approximate activation energies for the reference solution reactions (e.g. refs 14 and 32).

Since we viewed such estimates as crucial steps in any quantitative analysis of enzyme catalysis, it was clear that we could not take some of the traditional assumptions of physical organic chemists as a guiding rule (see, for example, refs 33−35). These assumptions included the perception that the nature of the transition states (TSs) in many reactions has been established experimentally, rather than being obtained from nonunique interpretation of the experimental findings. An instructive case in point is the unjustified assumption that linear free energy relationships can distinguish uniquely between associative and dissociative mechanisms of phosphate hydrolysis (see discussion in ref 34). With this in mind, we based our early estimates on a well-defined stepwise mechanism and on cross-information from different experiments. We also verified to ourselves that the stepwise and the concerted mechanisms gave similar barriers.

More recently, we started to use combinations of ab initio calculations and experiments to establish a consensus reference surface (e.g. refs 25, 26, and 36). Fortunately, in most cases, it has been found that the early estimates were reasonable. Here we can present, as an example, the estimate of the activation energy for the reference reaction in serine proteases, for which the early estimate was 25 kcal/mol[28] and the recent ab initio estimate is 26 kcal/mol.[25] Perhaps it is useful to point out that our early estimate has been criticized as being based on assumptions that contradict

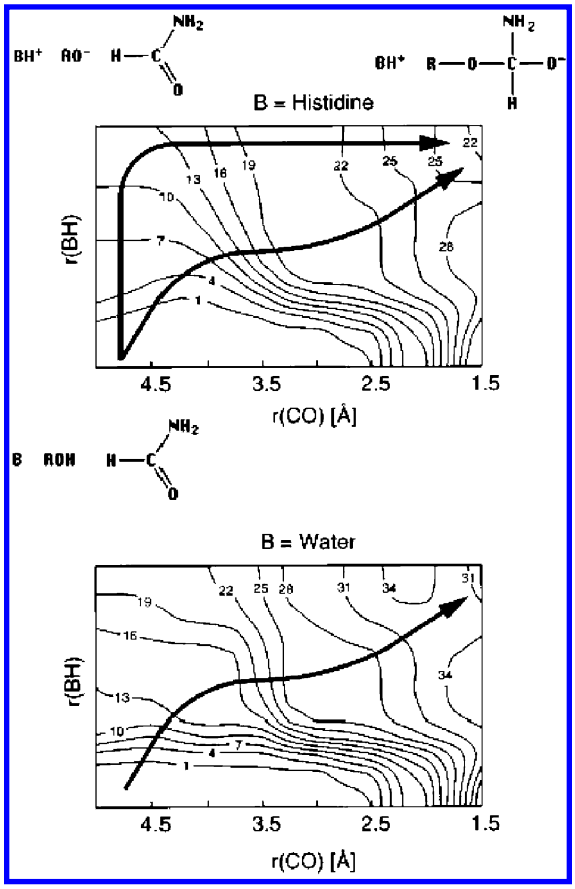


Figure 5. Schematic representation of the pK_a corrected free energy surfaces for the general base-catalyzed formation of the tetrahedral intermediate in the attack of methanol on formamide in aqueous solution calculated using the B3LYP$^+$+LD method. Top, histidine as a base; bottom, water as a base. The arrows represent the least energy path(s) on the given surface. (Reprinted with permission from ref 25. Copyright 2000 American Chemical Society.)

experimental facts[29,37] and, in particular, the experimental finding that the reaction is concerted rather than stepwise. However, as we pointed out repeatedly (e.g. ref 26), the observations that were considered as proofs for concerted paths have been simply interpretations of experiments, rather than experimental findings. For example, showing that a given isotope effect establishes a concerted path requires calculations of the isotope effect for both the concerted and the stepwise mechanisms, and no such calculation has ever been reported by the supporters of the concerted mechanism. Thus, it is significant to note that our careful ab initio study indicated that both the concerted and stepwise mechanisms have very similar energy[25] (see Figure 5). We also pointed out that studies that supported the concerted mechanism were actually gas-phase calculations.[37]

In addition to the above sources of information about the reference reaction, we can now make use of the experimental information provided during the past decade by Wolfenden and co-workers (e.g. refs 12, 38, and 39). Regardless of the above perspective, the main practical point of this section is the fact that reasonable combination of experimental and theoretical studies allows us to compile the benchmark given in Table 1. The table considers different types of enzymatic reactions, listing the energetics of the basic reaction in water, $[Rxn]_{w,w}$, the water reaction with the mechanism that occurs in the enzyme, $[Rxn]_{p,w}$, and the actual reaction in the enzyme. The results are also depicted in Figure 6, which provides a clear illustration of the enormous effect of the enzyme environment.

Table 1. Energetics of Different Enzymatic Reactions and the Corresponding Reference Reactions[a]

system[b]	$\Delta g^{\ddagger}_{w,w}$	$\Delta g^{\ddagger}_{p,w}$	$\Delta g^{\ddagger}_{cat}$	source[c]	$\Delta g^{\ddagger}_{p,w\rightarrow p,p}$ [d]
KI	27.0	22.1	11.2	224, 225	10.9 (15.8)
AR	22.5	22.5	14.8	226	5.7 (5.7)
CA	23.8	23.8	11.0	114	13.4 (13.4)
CM	24.5	24.5	15.4	90	9.0 (9.0)
trypsin	32.0	26.0	18.0	25, 28, 38	8.0 (14.0)
DhlA	27.0	27.0	15.3	95, 96	11.7 (11.7)
AP	27.5	27.5	15.2	109	12.0
Ras/G	27.5	27.5	16.1	112	11.4 (11.4)
TIM	26.4	28.4	14.0	227	14.4
Ach	36.0	29.5	13.5	13, 228	16.0 (22.5)
lysozyme	33.6	31.5	18.0	32	13.5 (15.6)
Rb (MI)	32.0	32.0	15.0	229	17.0 (17.0)
Rb (DI)	47.0	36.0	15.0	229	21.0 (32.0)
ATPase	37.0	37.0	14.8	126	22.2 (22.2)
Pol T7	32.0	38.2	15.0	113	23.2 (17.0)
ODCase	38.8	40.0	15.4	36, 203	24.6 (23.4)
Kf	36.0	46.4	19.0	230	27.4 (17.0)
SNase	36.0	51.5	14.9	39, 109, 111	33.9 (21.1)

[a] The table gives the activation barrier (in kcal/mol) for $[Rxn]_{w,w}$, $[Rxn]_{p,w}$, and the actual enzymatic reaction. [b] The following notation is used here: KI = ketosteroid isomerase; AR = aldose reductase; CA = carbonic anhydrase; CM = chorismate mutase; DhlA = haloalkane delahogenase; ATPase = F_1-ATPase; AP = alkaline phosphatase; Ras/G = Ras/GAP; TIM = triose phosphate isomerase; Ach = acetylcholine esterase; Rb(MI) = ribonuclease (monoionic intermediate); Rb (DI) = ribonuclease (diionic intermediate); Pol T7 = DNA polymerase T7; ODCase = orotidine 5′-monophosphate decarboxylase; Kf = the exonuclease activity of the Klenow fragment or DNA polymerase I; SNase = staphylococcal nuclease. [c] The indicated source includes a discussion and analysis of the reference reaction and the enzyme reaction. Note that, in several of the cases (e.g. TIM, SNase, and KI), we convert the reported $\Delta g^{\ddagger}_{cage}$ to $\Delta g^{\ddagger}_{w}$ by adding 2.5 kcal/mol (see ref 25 for discussion). For the reaction of ribonuclease, we consider both the diionic and monoionic mechanisms since it is not clear yet as to which mechanism is operational in the enzyme. The $[Rxn]_{p,w}$ reference reaction for Pol-T7 is taken with Asp as a base. The reference reaction for ATPase does not include the Mg^{2+} ion. For the lysozyme reaction, we consider the reaction with a carbonium ion intermediate. If the actual enzymatic reaction involves a nucleophilic attack by Asp 52, then the reference reaction still involves a major carbonium character and the activation barrier is similar to that estimated for the carbonium mechanism. [d] The catalytic effects relative to $[Rxn]_{p,w}$ and $[Rxn]_{w,w}$ are given with and without parentheses, respectively.

The table provides a semiquantitative illustration of the environmental effect of the enzyme. Our new scale removes the binding contributions to $\Delta g^{\ddagger}_{p}$ as well as the effect of considering the $[Rxn]_{p,w}$ reference reaction. As is clear from the table, we have cases with enormous environmental effects, which are even much larger than the "upper limit" of 15 kcal/mol limit, proposed in ref 5, based on the unjustified assumption that the binding of substrates provide a way for establishing the limit of TS binding energies.

We would like to clarify at this point that our aim is not the compilation of a very large number of enzymatic reactions, but to establish a meaningful and reliable benchmark. Our point is that the analysis of the reference solution reaction can require a significant computational effort and we prefer to have reliable information about a number of reactions, rather than a massive collection of unverified data. Of course, our table can easily be extended, but it already provides what we believe is a sufficiently large representation of different catalytic effects and a benchmark that can easily be used to verify or to exclude different catalytic proposals.

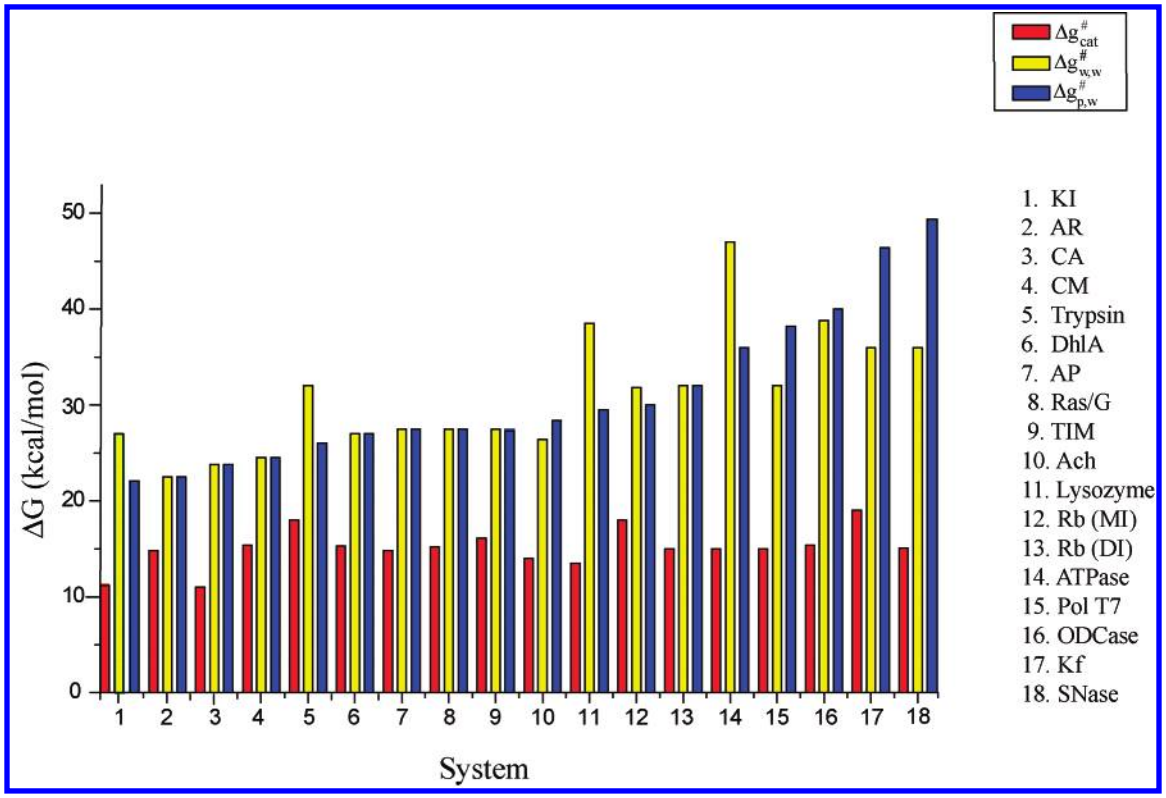


Figure 6. Activation free energies of representative enzymatic reactions ($\Delta g^{\ddagger}_{cat}$) and the corresponding reference solution reactions for the same mechanism as the enzymatic reaction ($\Delta g^{\ddagger}_{p,w}$) and the actual mechanism in water ($\Delta g^{\ddagger}_{w,w}$). The notation of the different enzymatic reactions is defined in Table 1.

After establishing the above benchmark, we are ready to explore the origin of its enormous catalytic effects. In doing so, it is important to clarify that, in contrast to the implication of the recent proposal of ref 5, the catalytic power of enzymes is not related directly to the binding power of proteins. That is, we are not dealing here with absolute binding, which may involve many different contributions, but rather with the relative binding of the reactant state (RS) and the TS. From this perspective, it may be hard to see how we can get a difference of even a few kilocalories per mole between the binding energies of these two states (for example, hydrophobic effects cannot give large contributions to catalysis, but they can contribute in a major way to binding energies). Yet, the difference in binding energy between the RS and TS is enormous. Thus, the elucidation of the origin of this difference has remained one of the most exciting secrets in biochemistry. More specifically, most proposals that were introduced to account for the catalytic effect of enzymes (e.g., strain, entropy, desolvation, tunneling, and covalent effects) cannot account for more than a few kilocalories per mole of the catalytic effect (e.g., see discussion in refs 1 and 27 and section 5). Thus, it is clearly important to use computer simulations and to see what contributions account for $\Delta\Delta g^{\ddagger}$.

The above issues will be addressed in sections 4−6 after a brief introduction of our theoretical approaches.

3. Evaluating Activation Free Energies in Enzyme Active Sites by the EVB and MO QM/MM Methods

To examine the origin of the catalytic power of enzymes and to discriminate between different catalytic proposals, it is essential to have quantitative methods for calculating the rate constant of a reaction starting from the structure of the given enzyme. Since the key factor in such calculations is the activation free energy,[17] the main challenge is the development and validation of methods for calculating activation free energies. Any such method requires evaluating the potential energy surface that connects the reactant and product states and finding the activation free energy for reaching the TS. Combined quantum mechanical/molecular mechanical (QM/MM) methods provide a generic way of obtaining potential surfaces and, in principle, activation free energies of chemical processes in enzymes. This approach, introduced in 1976,[40] has gained popularity in recent years and has been used in a variety of forms (for reviews, see refs 10, 27, and 41). However, implementation of rigorous, ab initio QM/MM approaches in quantitative calculations of activation free energies is still extremely challenging. Nevertheless, significant progress is starting to emerge from recent works. (e.g. refs 27 and 42−47) Furthermore, semiempirical QM/MM studies with reasonable potential of mean force (PMF) calculations, and in some cases even with least energy paths, can be used to assess the validity of some catalytic proposals (e.g. refs 48−50).

Despite the progress in ab initio QM/MM evaluations of activation barriers, we prefer to focus here on the less rigorous empirical valence bond (EVB) method,[1,32] since it provides what is probably the most effective available way for quantifying the catalytic effect and determining its origin. The EVB method is a QM/MM approach, which describes the system with two or more resonance states (or, more precisely, diabatic states) corresponding to classical valence-bond structures. These basis states are mixed to describe the reactant intermediate states.

As an example, for an S_N2 reaction of the form

$$X^- + CH_3Y \rightarrow XCH_3 + Y^-, \quad (2)$$

one can use diabatic states of the forms

$$\phi_1 = X^- \; CH_3{-}Y$$

$$\phi_2 = X{-}CH_3 \quad Y^- \quad (3)$$

The potential energies of these states (H_{11} and H_{22}) and the mixing term (H_{12}) are represented by the Hamiltonian matrix elements

$$H_{ii} = \epsilon_i = \alpha^{\,i}_{\text{gas}} + U^{\,i}_{\text{intra}}(\mathbf{R},\mathbf{Q}) + U^{\,i}_{\text{inter}}(\mathbf{R},\mathbf{Q},\mathbf{r},\mathbf{q}) + U^{\,i}_{\text{solvent}}(\mathbf{r},\mathbf{q}) \quad (4a)$$

$$H_{ij} = A \exp(-a|\Delta R'|) \quad (4b)$$

Here **R** and **Q**, respectively, represent the atomic coordinates and charges of the reactants or products (the "solute") in the diabatic states, and **r** and **q** are the coordinates and charges of the surrounding water or protein (the "solvent"). $\alpha^{\,i}_{\text{gas}}$ is the energy of the *i*th diabatic state in the gas phase, where all the fragments are taken to be at infinity; $U^i(\mathbf{R},\mathbf{Q})$ is the intramolecular potential of the solute system (relative to its minimum) in this state; $U^i(\mathbf{R},\mathbf{Q},\mathbf{r},\mathbf{q})$ represents the interaction between the solute atoms and the surrounding solvent atoms; and $U^i(\mathbf{r},\mathbf{q})$ represents the potential energy of the solvent.

The ϵ_i's given by eq 4a form the diagonal elements (H_{ii}) of the EVB Hamiltonian ($\mathbf{H}_{\text{EVB}}$). The off-diagonal elements of the Hamiltonian (H_{ij}) are either assumed to be constant or are represented by an exponential function of the distances between the reacting atoms. In the present case, we express H_{ij} as a function of the difference between the X—C and C—Y bond lengths ($\Delta R'$ in eq 4b), using parameters (A and a) that are adjusted to fit either quantum calculations or experiments. The H_{ij} elements are assumed to be the same in the gas phase, in solution, and in the protein. The adiabatic ground-state energy (E_g) and the corresponding eigenvector ($\mathbf{C}_g$) are obtained by solving the secular equation,

$$\mathbf{H}_{\text{EVB}}\mathbf{C}_g = E_g\mathbf{C}_g \quad (5)$$

To express the adiabatic energy surface of the solute−solvent system, it is useful to define a generalized reaction coordinate as the energy gap between the diabatic reactant and product EVB states:

$$x = \Delta\epsilon_{1,2} = \epsilon_2 - \epsilon_1 \quad (6)$$

This coordinate can be divided into a solute coordinate, *R*, for internal bonds of the reacting EVB structures and a solvent coordinate, *S*, for interactions of the solute with the solvent. "Solvent" here is used in a general sense to refer to the surroundings of the reacting atoms in either the enzyme or the solvent.

The simplicity of the EVB formulation makes it relatively straightforward to obtain analytical derivatives of the potential surface by using the Hellmann−Feynman theorem for eq 5 and, thus, to sample the EVB energy surface by molecular dynamics (MD) simulations. In principle, running MD trajectories on the EVB surface of the reactant state can provide the free energy function (Δg) that is needed to calculate the activation free energy ($\Delta g^{\ddagger}$). However, since trajectories on the reactant surface will reach the TS only rarely, it is usually necessary to run trajectories on a series of potential surfaces ("mapping" potentials) that drive the system adiabatically from the reactant to the product state.[51] In the simple case of two diabatic states such as those of eq 3, the mapping potential (ϵ_m) can be written as a linear combination of the reactant and product potentials, ϵ_1 and ϵ_2:

$$\epsilon_m = (1 - \lambda_m)\epsilon_1 + \lambda_m\epsilon_2 \quad (7)$$

where λ_m changes from 0 to 1 in $n + 1$ fixed increments ($\lambda_m = 0/n,\ 1/n,\ 2/n,\ \ldots,\ n/n$).

The free energy ΔG_m associated with changing λ from 0 to m/n can be evaluated by a free-energy perturbation (FEP) procedure (see, e.g., chapter 3.3.2 in ref 1). The free energy functional that corresponds to the adiabatic ground-state surface E_g then is obtained by the FEP-umbrella sampling (FEP-US) method,[1,51] which can be written as

$$\Delta g(x') = \Delta G_m - \beta^{-1} \ln\langle \mathrm{d}(x - x') \exp\{-\beta[E_g(x) - \epsilon_m(x)]\}\rangle_m \quad (8)$$

In this expression, ϵ_m is the mapping potential that keeps the reaction coordinate x in the region of x', $\langle\cdots\rangle_m$ denotes an average over an MD trajectory on this potential, $\beta = (k_BT)^{-1}$, k_B is the Boltzmann constant, and T is the temperature. If the changes in ϵ_m are sufficiently gradual, the free energy functionals $\Delta g(x')$ obtained with several values of m overlap over a range of x', and patching together the full set of $\Delta g(x')$ gives a complete free energy curve for the reaction.

The FEP-US approach can also be used to obtain the free energy functionals of the individual diabatic states. For example, the free energy of the reactant state (Δg_1) is expressed as

$$\Delta g_1(x') = \Delta G_m - \beta^{-1} \ln\langle \mathrm{d}(x - x') \exp\{-\beta[\epsilon_1(x) - \epsilon_m(x)]\}\rangle_m \quad (9)$$

The diabatic free energy profiles of the reactant and product states (the free energy functionals) represent microscopic equivalents of the Marcus parabolas in electron-transfer theory.[52] The intersection of these free energy functionals provides a quantitative estimate of the reorganization energy, which will play a key role in our considerations (see section 4.2).

The natural picture of intersecting electronic states provided by the EVB treatment is particularly useful for exploring environmental effects on chemical reactions in condensed phases.[53] The ground-state charge distribution of the reacting species (solute) polarizes the surroundings (solvent), and the charges of each resonance structure of the solute then interact with the polarized solvent.[1] This coupling enables the EVB model to capture the effect of the solvent on the quantum mechanical mixing of different states of the solute. For example, if ionic and covalent states are used to describe the solute, preferential stabilization of the ionic state by the solvent will give the adiabatic ground state more ionic character. This allows one, for example, to obtain a very well-defined separation of covalent (charge transfer) and electrostatic effects and, thus, to analyze in a clear way some

covalent hypotheses (see sections 5.7−5.8). In addition, the EVB method lends itself to proper configurational sampling and converging free energy calculations, which makes it possible to evaluate nonequilibrium solvation effects.[17]

The EVB and other QM/MM methods allow one to simulate chemical reactions in enzyme active sites and solution and to reproduce the corresponding activation barriers. Once the calculated change in activation barriers, $\Delta g^{\ddagger}_{cat} - \Delta g^{\ddagger}_{p}$, reproduces the observed catalytic effect, we may start exploring the factors that contribute to this effect. Studies that moved along this line will be considered below.

4. Electrostatic Contributions of Preorganized Active Sites

In our examination of the origin of enzyme catalysis, we will start by focusing on the proposal[14,54] that the catalytic power of enzymes (the reduction in $\Delta\Delta g^{\#}$) is almost exclusively due to electrostatic effects.

With the current insight, it might be argued that electrostatic effects must have been the most obvious candidates for explaining enzyme catalysis. However, careful studies in the early stages of the field have basically excluded this possibility. That is, early experiments with model compounds in solution (e.g. refs 55 and 56) that explored the role of electrostatic effects (by introducing charged groups to stabilize the TS charge distribution, as illustrated schematically in Figure 7) concluded that such effects must be small (e.g., see refs 55 and 56). Similarly, phenomenological attempts to estimate the magnitude of electrostatic contributions to catalysis[57] also indicated that such effects are small. Thus, it was assumed more or less uniformly (at least in studies that attempted to quantify the catalytic effect) that electrostatic effects do not play a very important role. The problem has, however, been that physical organic chemistry experiments in solution might have been rather irrelevant to an enzyme active site. Similarly, phenomenological attempts to estimate the strength of electrostatic effects in proteins have been very problematic since it is almost impossible to assess the dielectric effects in the protein without a proper computational model. In this respect, it is also important to clarify that the view expressed by the pioneering work of Jencks[58] did not consider electrostatic stabilization of the TS as a major catalytic effect. In fact, Jencks focused on the role of electrostatic and desolvation effects as the price to be paid for substrate "destabilization". Thus, it appears that the microscopic electrostatic study in 1976 by Warshel and Levitt[40] provided the first quantitative hint that electrostatic effects can play a major role in enzyme catalysis.

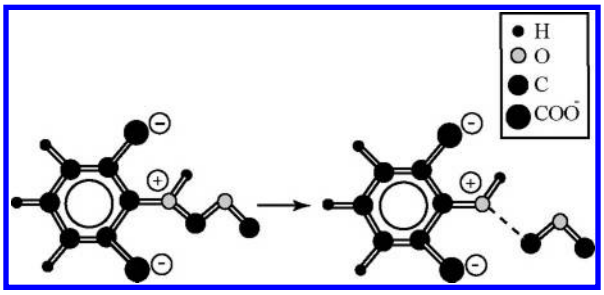


Figure 7. Type of model compounds that were used to estimate the electrostatic stabilization in lysozyme. Such molecules do not show large rate acceleration due to electrostatic stabilization of the positively charged carbonium transition state. However, the reaction occurs in solution and not in a protein-active site, and the dielectric effect is expected to be very different in the two cases.

Before we move to specific discussion, it is important to emphasize that the definition (and, more importantly, the meaning) of "electrostatic catalysis" has been clarified long ago (e.g. refs 40, 59, and 60). It includes the effects of the protein charges, permanent dipoles (residual charges), induced dipoles (polarizability), and, of course, the solvation by bound water molecules. It does not include van der Waals strain effects, charge-transfer covalent interactions, orientational entropy, and dynamical effects. This clarification should be kept in mind when considering the argument that all quantum chemistry is electrostatic. What counts is what was meant by the electrostatic proposal and by other proposals before it becomes clear that electrostatic effects are crucial for catalysis.

4.1. General Studies

Before we consider the growing theoretical support for the role of electrostatic stabilization in catalysis, it is important to comment about the insight that emerged from mutation experiments starting from around 1984 (e.g., refs 2 and 61−66). These mutation experiments have provided major insights, and in many cases, they pointed toward the importance of electrostatic effects. More recent works have added extensive support to this view (e.g., refs 67 and 68). However, since the catalytic effect reflects the overall effect of the enzyme active site, it has been very hard to reach unique conclusions about the overall electrostatic effect. Furthermore, even when a mutation of an ionized group to a nonpolar group leads to a large reduction in k_{cat}, it has been close to impossible to determine experimentally whether this is an electrostatic effect or some other factor (an excellent example is the D102N mutation of trypsin,[69] discussed in ref 70).

With the above background in mind, it seems to us that the use of QM/MM and related approaches provides what is perhaps the best way to convert the structures of enzyme active sites to catalytic contributions. In fact, since 1976, there have been a growing number of MO-QM/MM and EVB calculations that identify electrostatic effects as a key factor in enzyme catalysis. This trend has moved the field gradually from a stage of qualitative statements (e.g., see ref 71) to more quantitative conclusions. Here it is useful to consider the studies summarized in Table 2. This table only considers studies that actually examined the catalytic effect rather than general QM/MM studies. Thus, the studies in the table include both EVB and MO-QM/MM calculations. Full analysis of the electrostatic effects has been provided at present mainly from EVB studies, since this requires not only calculations of the activation free energy in enzyme and solution but also evaluation of the electrostatic contribution to the binding free energy of the RS and TS. However, MO QM/MM approaches have studied the electrostatic interaction energies (e.g., refs 49 and 72) and even reported systematic progress in evaluating the change in the electrostatic free energy along the reaction coordinate (e.g., ref 73). Furthermore, many of the EVB studies provide the solvent reorganization energy and demonstrate that the contribution accounts for a major part of the catalytic effect (see below). Here, it is useful to mention recent attempts to estimate the reorganization energies by MO-QM/MM approaches (e.g., ref 74), but these only considered the change in the environment MM energy rather than systematic calculations using eq 9 or related linear response approximation (LRA) treatments. Unfortunately, the change in the very large total

Table 2. Calculation of Enzymatic Reactions and Analysis of Catalytic Contributions[a]

system	method	reliability[b]	$\Delta g^{\ddagger}_{p,w\rightarrow p,p}$	origin	mutations[c]	ref
lysozyme	MO-QM/MM	∼		no strain	el	40
	EVB/PDLD	∼	+			32
	EVB/PDLD	∼		el	el	70
serine protease	EVB	+	+		el	101
	EVB	+			el	165
	MO-QM/MM	+	+		el	73
CM	MO-QM/MM	+	+	el		76, 195
	EVB	+	+	el		90
	MO-QM/MM	∼			el	49
DhlA	EVB	+	+	el		95, 96
	MO-QM/MM	+	+			83
	MO-QM/MM	+	+			74
ODCase	EVB	+		el		36
	MO-QM/MM	+ (−)[d]			el_{GSD}[c]	50
ribosome	EVB	+	+	el[d]		98
	EVB	+	+	el		97
Ach	PDLD/S	∼	+	el		228
	MO-QM/MM	+	−		el	231
	EVB	+				232
ADH	EVB	+	+	λ_{el}		17
	EVB	+				186
	MO-QM/MM	∼			el	233
aldosereductase	EVB	+	+	λ_{el}		226
TIM	EVB	+	+	λ_{el}		227
	MO-QM/MM	−	∼			234
	MO-QM/MM	+			el	235
	MO-QM/MM	−	?			236
	EVB	+		no strain		77
P450	MO-QM/MM	+ (−)	+		el	237
	MO-QM/MM	(+) (−)	+		el	80
enolase	MO-QM/MM	+	−	−	el	238
tyrosine phosphatase	EVB	+	+		el	239
	MO-QM/MM	+				240
CA		+	+	el_{metal}		114
SNase		+	+	el_{metal}	el	111
Pol T7		+	+			113
Kf		+	+	el_{metal}		230
Ras/Gap	EVB	+	+	el	el	8
LDH	EVB	+	+	λ_{el}		177
	MO-QM/MM	+				241
xylose isomerase	MO-QM/MM	+				
KS	EVB	+	+	el		224
Glx1	EVB	+	+	el_{metal}		77
O-methyltrasperase	MO-QM/MM	+	+	el		72
β-lactomase	MO-QM/MM	∼			el	242
B_{12}[e]	MO-QM/MM	∼[e]	+	strain + el		145
	EVB	+	+	el		152

[a] Energies in kcal/mol. "el" designates the electrostatic effect, and λ_{el} denotes an identification of changes in the "solvent" reorganization energy as a key catalytic effect. [b] + and ∼ designate, respectively, fully quantitative and qualitative conclusions. Within this definition, we consider as fully reliable calculations those studies that involve free energy calculations and have their results calibrated with solution studies. [c] Mutation studies in some cases include estimates of the contributions of different residues without performing these actual mutations. Such assessments frequently overestimate the electrostatic effect, because microscopic studies usually drastically underestimate the dielectric effect for charge−charge interactions (e.g., see ref 75). [d] The study by Gao and co-workers[50] gave reasonable activation energies and reproduced the catalytic effect, but their attempt to evaluate the binding energy of the RS and the TS by FEP calculations gave similar energies and, thus, could not reproduce the catalytic effect or assess its origin. The incorrect thermodynamic cycle study is analyzed in section 5.6. [e] B_{12} designates the reaction of B_{12}-dependent enzyme. The possible difficulty of evaluating the free energy contribution by different methods is discussed in section 5.1.

MM energy during the reaction is a rather unstable quantity, which is hard to evaluate in a quantitative way.

In many cases, MO-QM/MM calculations can provide clear indications that the electrostatic effects play a major role in catalysis by simply evaluating the contributions of different residues to the activation barrier. Unfortunately, this type of "mutational" analysis is frequently very qualitative since the simulations do not provide a sufficient dielectric screening. The underestimation of the screening effect is quite problematic, when one deals with ionized protein residues (see discussion in ref 75). Moreover, a proper analysis of the catalytic effect should explore the overall electrostatic contribution of the active site rather than just the contribution of some residues. In any case, we also list in Table 2 the studies that explore the electrostatic effects of different residues. In a few cases (e.g., ref 76) we already have QM/MM studies that eliminated step by step the electrostatic contributions of the enzyme environment and thus established the importance of the overall electrostatic effect.

Our considerations of electrostatic effects will also include the effect of metal ions, representing them by electrostatic models. Although the justification for such a treatment will be given in section 4.4, we would like to note the instructive study by Åqvist and co-workers,[77] who considered the effect

of different metal ions and obtained very similar results (in agreement with experimental observations). This provides strong support to analysis that considers the effect metal ion as an electrostatic effect (see also section 4.4).

One can, of course, point to instructive studies that included only a limited part of the enzyme and reproduced the observed barrier, in particular, in cases of metal centers or radical reactions.[78,79] However, these cases do not involve a significant effect of the enzyme active site (except in assembling the reacting system), and thus, the same cluster would work as well in water.

It may also be useful to consider here recent studies of P450, which described the enzyme catalytic effect as a "chameleon" effect[80] and which also provided further support to our electrostatic concept. That is, the enzyme polar groups and, in particular, the N−H bonds from Leu358, Gly359, and Gln360 are thought to change the electron distribution in the reacting system and thus to control the specificity.[81] This is basically another example of the interaction between the electrostatic field from the enzyme and the reacting cofactor (see a related early electrostatic study of the control of oxygen binding to hemoglobin).[82]

Some QM/MM and other related studies (see below) do not support the idea of electrostatic transition-state stabilization. However, at present, all of these studies have suffered from significant inconsistencies. The key examples are as follows: (i) works that attributed the catalysis to desolvation or the ground-state electrostatic destabilization [These works did not consider the actual binding of the TS and RS (e.g., refs 83 and 84) and could not reproduce the actual catalytic effect by the binding calculations (e.g., refs 50, 84, and 85).]; (ii) works that could not reproduce the catalytic effect without the use of entirely inconsistent entropic calculations that included major overestimates based on gas-phase vibrational analysis (e.g., ref 86) (also see discussion in ref 87); and (iii) the NAC proposal of Bruice and co-workers,[88] also supported by other groups (e.g., ref 89) (which is, in fact, an electrostatic transition-state stabilization (TSS) effect, as will be discussed in section 5.3; see also ref 90). Finally, it's important to comment here on the idea[91] that enzyme catalysis is due to reactant-state destabilization (RSD), which is formulated in terms of a decrease in the enzyme self-energy upon moving to the TS. As will be shown in section 5.6, this idea is based on inconsistent considerations.

It is useful to consider here a recent attempt by Kraut et al.[246] to show that the electrostatic effect cannot provide the major contribution to enzyme catalysis. The authors found a weak correlation between binding energies of phenolate ions to the oxyanion hole of ketosteroid isomerase (KI) and the delocalization of the phenolate's hydrogen bonding (as estimated from NMR shifts). This correlation was assumed to provide an experimental tool for assessing the importance of electrostatic energies in enzyme catalysis. The finding of a very small change in binding energy for a significant change in delocalization was interpreted as evidence that electrostatic contributions do not play a major role in KI and presumably in other enzymes. However, in this case, as in many other cases, there is a risk of confusing an interpretation of experimental fact with a unique energy-based analysis. The most careful estimates of the catalytic effect of KI have found that it is almost entirely due to electrostatic preorganization effects.[224] The experimental correlation described by Kraut et al. does not provide a way to estimate the actual electrostatic contribution or, for that matter, to quantify any other contribution. The authors simply postulated that the electrostatic effect must be correlated with the presumed localization of the charge in the isolated phenolate, and they tried to deduce from it the corresponding electrostatic contribution. There are many problems with this approach. First, more than half of the binding energy is due to nonpolar contributions and not due to electrostatic effects, whereas there are no nonpolar contributions in the correct catalytic cycle of the actual substrate. Second, the correlation between the pK_a of the phenolate and the charge on the oxygen is problematic, as can be established by ab initio calculations. Third, a less localized charge on the oxygen will also have smaller electrostatic stabilization (solvation) in the reference solvent. Fourth, while the changes in the NMR shifts are probably correlated in part with the changes in delocalization of the hydrogen bond to Tyr 16 (rather than with the delocalization on the isolated phenolate), the electrostatic contributions to catalysis are not correlated with the degree of delocalization of the hydrogen bond but with the stabilization of the lower energy localized state, which is then mixed with the other state (see e.g. ref 59). One way to see this point is to consider enzyme catalysis of S_N2 reactions where the charge is completely delocalized in the TS and yet the catalytic effect is enormous and due entirely to the electrostatic reorganization effects (e.g. ref 95). At present the most effective way of analyzing the electrostatic contributions of hydrogen bonding in oxyanions is to use a valence bond type description, as was done in refs 59 and 224. When this is done, one finds that the preorganization of the hydrogen bond is the main source of catalysis and that this contribution is not correlated with charge delocalization but with the folding of the active site.

To summarize this section, it seems to us that careful considerations of the works mentioned in Table 2 as well as consistent attempts to identify the origin of large catalytic effects point toward the conclusion that electrostatic effects are the key factors (this issue will be emphasized and quantified further in the following section).

4.2. Quantifying the Source of Electrostatic Contributions to Catalysis

The studies reported above provide general support to the electrostatic proposal. A more quantitative analysis is provided in Table 3. As seen from the table, we have clear examples of specific cases where most of the catalytic effect is due to electrostatic interactions. What remains to be established is that these effects are associated with TS stabilization and to examine what is the reason for the ability of the protein to provide such large effects. These issues can be explored by using the LRA expression[92] for the TS and

Table 3. Electrostatic Contribution to the Catalytic Effects of Specific Enzymes[a]

system	$(\Delta\Delta g^{\ddagger}_{tot})_{calc}$	$(\Delta\Delta g^{\ddagger}_{elect})_{calc}$	$(\Delta\Delta g^{\ddagger}_{cage})_{calc}$	$\Delta\Delta g^{\ddagger}_{obs}$	ref
DhlA	11.6	8[b] (8)	2.3	11.7	95, 96
CM	10.3	8 (14)	0.0	9.1	90
ODCase	19.0	17[c]		23.0	36
ribosome	8.0	− (8)	0.0	6.0	97

[a] The table compares the total calculated catalytic effect and the corresponding electrostatic contribution. All energies are given in kcal/mol. [b] Obtained by taking the calculated catalytic effect of ref 95 and multiplying it by the percent contribution obtained in ref 96. The values within parentheses were obtained by the LRA approach. [c] Obtained by FEP calculations of the binding energy of the RS and TS.

the RS. For the TS, we have

$$\Delta G(Q^{\ddagger}) = 0.5(\langle U(Q{=}Q^{\ddagger}) - U(Q{=}0)\rangle_{Q=Q^{\ddagger}} + \langle U(Q{=}Q^{\ddagger}) - U(Q{=}0)\rangle_{Q=0}) = 0.5(\langle \Delta U\rangle_{Q^{\ddagger}} + \langle \Delta U\rangle_{0}) \quad (10a)$$

$$\lambda^{\ddagger} = 0.5(\langle \Delta U\rangle_{0} - \langle \Delta U\rangle_{Q^{\ddagger}}) \quad (10b)$$

where U is the solute−solvent interaction potential, Q designates the residual charges of the solute atoms with $Q^{\ddagger}$ indicating the TS charges, and $\langle \Delta U\rangle_{Q}$ designates an average over configurations obtained from an MD run with the given solute charge distribution. The quantity, λ, in eq 10b is the reorganization energy for solvation of the TS, whose nature will be discussed below. The first term in eq 10 is the above-mentioned interaction energy at the TS, where $Q = Q^{\ddagger}$, which is similar in the enzyme and in solution. The second term expresses the effect of the environment preorganization. If the environment is randomly oriented toward the TS in the absence of charge (as is the case in water), then the second term is zero and we obtain

$$\Delta G(Q^{\ddagger})^{w}_{sol} = \frac{1}{2}\langle \Delta U\rangle_{Q^{\ddagger}} \quad (11)$$

where the electrostatic free energy is half of the average electrostatic potential.[93] However, in the preorganized environment of an enzyme, we obtain a significant contribution from the second term and the overall $\langle \Delta U\rangle_{Q}$ is more negative than that in water. This extra stabilization is the catalytic effect of the enzyme. Another way to see this effect is to realize that in water, where the solvent dipoles are randomly oriented around the uncharged form of the TS, the activation free energy includes the free energy needed to reorganize the solvent dipoles toward the changed TS. On the other hand, the reaction in the protein costs less reorganization energy since the active site dipoles (associated with polar groups, charged groups, and water molecules) are already partially preorganized toward the TS charge.[14] The reorganization energy is related to the well-known Marcus' reorganization energy, but it is not equal to it. More specifically, the Marcus' reorganization energy[94] is related to the transfer from the reactant to the product state, while here we deal with charging the TS. The conceptual and practical differences (see ref 17 for a detailed discussion) are demonstrated, for example, in Figure 8, where we consider two cases. In the first case (Figure 8a), $\Delta G_0 \cong 0$ and the catalytic effect is directly related to the difference between the Marcus reorganization energy for the enzyme and the solution reactions. In the second case (Figure 8b), $\Delta G_0 > 0$ and the catalytic effect is associated with the reduction of ΔG_0. This is also done by the preorganization effect (the second term in eq 10), but now we are talking about the reorganization energy along the solvent coordinate with respect to the solvation of the product state rather than along the reaction coordinate.

Regardless of the above clarification, it is almost always true that the catalytic effect is associated with the reduction of the Marcus reorganization energy so that $\lambda_p \leq \lambda_w$. This point and the related role of preorganization in the electrostatic environment are demonstrated schematically in Figure 9 and quantified for the case of DhlA[95,96] in Figure 10 (based on eq 10 for the RS and TS). An LRA analysis is given in Table 4 for CM and in Table 5 for ribosome and DhlA. As

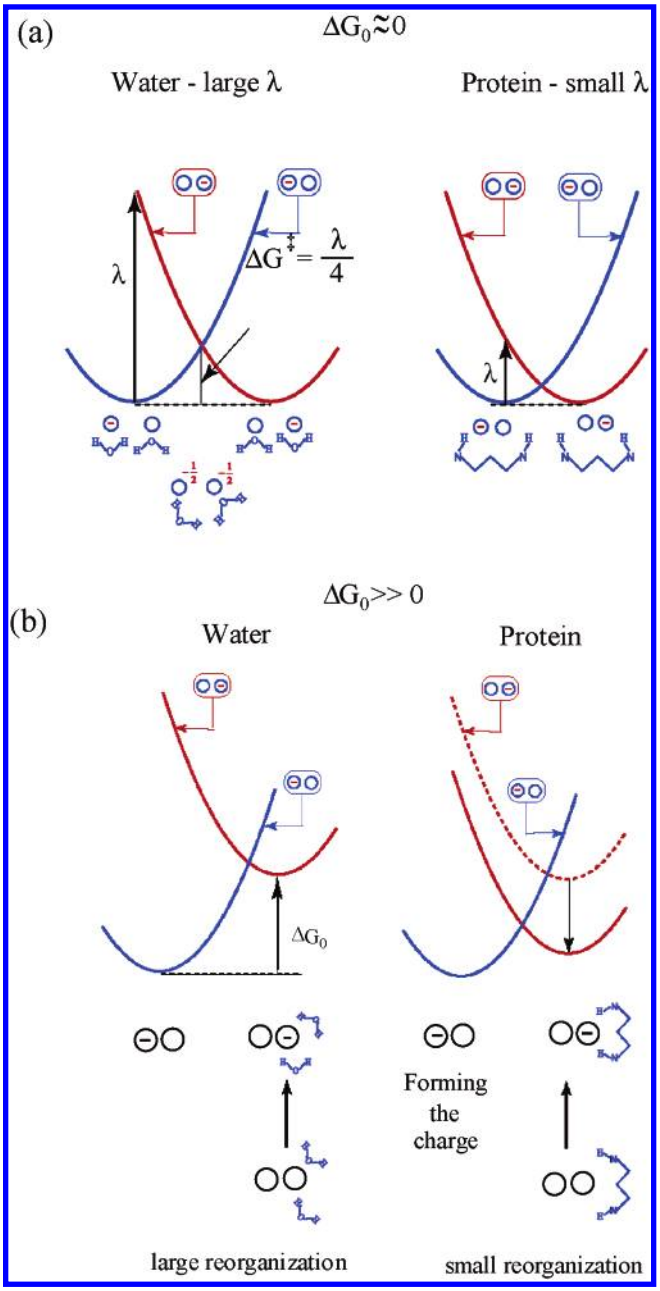


Figure 8. Illustration of the nature of the preorganization effect in two limiting cases. (A) In the limit when $\Delta G_0 = 0$, the enzyme has a smaller λ, since its dipoles are already partially preorganized toward the TS charge distribution. This corresponds to a reduction of the Marcus "reorganization energy". (B) In the case when $\Delta G_0 \gg 0$, the enzyme dipoles are preorganized toward the product charge distribution. In this way, the preorganization helps to increase the solvation of the product state.

seen from these cases and other related studies, the catalytic effect appears to be associated mainly with the electrostatic stabilization of its TS, and a large part of the effect is associated with the preorganization contribution. Interestingly, even in the case of peptide bond formation by the ribosome (which constitutes a very early stage in the evolution of biocatalysis), it has been found that the preorganization effect provides the major catalytic effect.[97,98,247]

The correlation between the reorganization energy and the catalytic effect has been explored recently (Liu and Warshel, in preparation) in a study of the effect of mutations in dihydrofolate reductase (DHFR). This is a very interesting benchmark, since the effects of mutations were used as

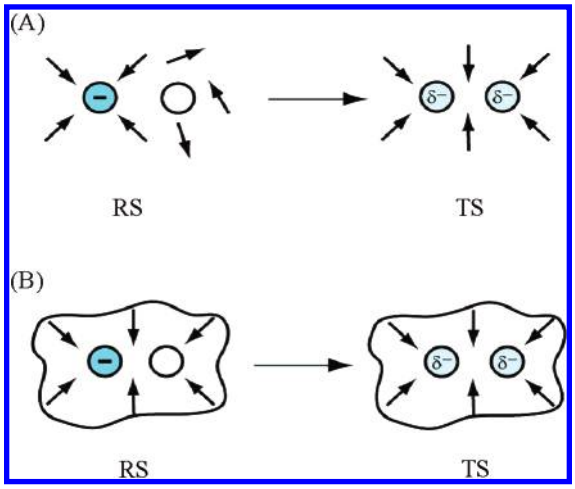


Figure 9. Schematic demonstration of the reorganization of the environment dipoles in an S_N2 reaction: (A) in water; (B) in an enzyme active site.

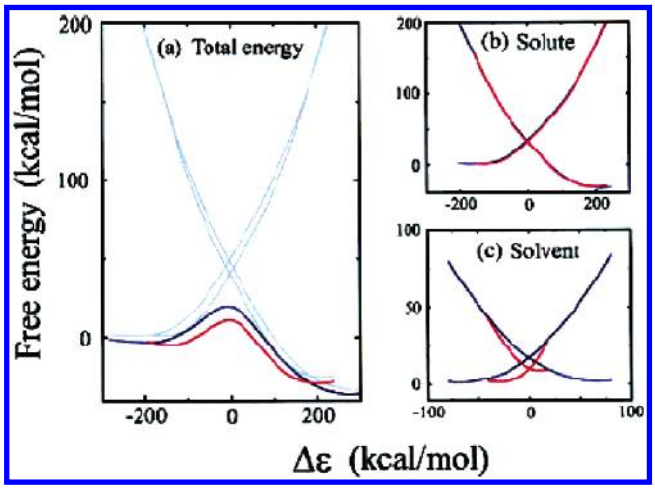


Figure 10. Description of the free energy surface of the S_N2 reaction step in DhlA (red lines) and in water (blue lines) in terms of generalized solute and solvent coordinates: (a) total free energy function for the enzyme and the water system (red and blue, respectively); (b) solute and (c) solvent components of the free energy function. As seen from the figure, the difference between the free energy surfaces of the enzyme and water reaction is due to the difference along the solvent coordinate (which reflects the change in λ_Q). (Reprinted with permission from ref 95. Copyright 2004 American Chemical Society.)

Table 4. LRA Analysis of the Electrostatic Solvation Contributions to the Binding of the RS and TS in CM[a]

	water		protein	
	RS	TS	RS	TS
$\langle \Delta U \rangle_Q$	−388.8	−403.1	−369.0	−402.8
$\langle \Delta U \rangle_0$	0.0	0.0	−66.2	−74.4
ΔG_{LRA}	−194.4	−201.5	−217.6	−238.6

[a] All energies are taken from ref 90 and are given in kcal/mol. $\langle \Delta U \rangle$ designates the average of the electrostatic interaction between the substrate and its surroundings (water and protein). $\langle \Delta U \rangle_Q$ and $\langle \Delta U \rangle_0$ designate the corresponding averages over a potential surface that includes a fully charged substrate and a nonpolar substrate, respectively. The calculated energies are converted to "solvation" energies by subtracting the corresponding values of $\langle \Delta U \rangle_Q$ in water.

evidence of the catalytic effect of correlated motions (see section 5.4). Now, as seen from Figure 11, there is a good correlation between the reorganization energy and the catalytic effect.

Table 5. Solvation Energies[a] for the Enzyme and Water Reference Reaction in the Reactant State (RS) and the Transition State (TS) of DhlA

	water reference reaction		enzyme reaction	
	RS	TS	RS	TS
$\langle U \rangle_Q$	−158.5	−114.2	−129.7	−102.4
$\langle U \rangle_0$	2.9	4.4	−62.0	−59.1
ΔG_{solv}	−77.8[b]	−54.9	−95.9[b]	−80.8

[a] All energies are taken from ref 95 and are given in kcal/mol. [b] As can be seen from these results, the reacting fragments are solvated better in the enzyme than in the water reference reaction, which shows that the primary function of the enzyme is not to desolvate the substrate. Instead, the transition state is better solvated in the enzyme than in the water reaction.

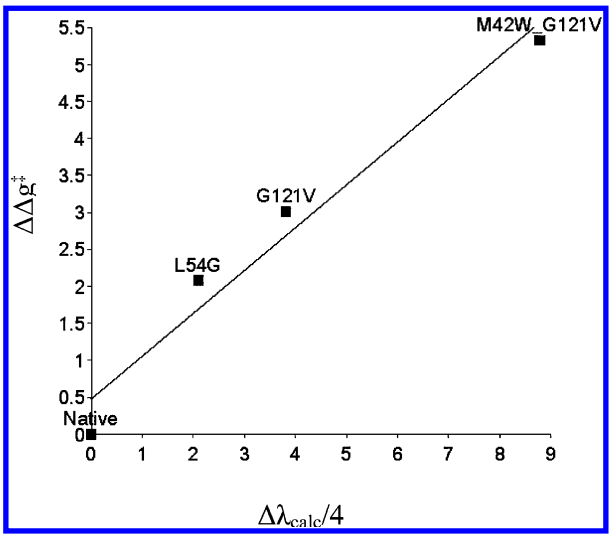


Figure 11. Correlation between the calculated reorganization energy and the observed mutational effects in DHFR (mutational effects are taken from refs 158, 243, and 244).

It might be important at this point to clarify the unique feature of our preorganization proposal. Some workers (e.g., refs 99 and 100) have suggested that the reduction of the protein reorganization energy will result in catalysis according to the Marcus relationship. However, these workers could only rationalize such a reduction due to the existence of a nonpolar active site. Unfortunately, protein active sites are polar (instead of being nonpolar) and having a nonpolar active site would drastically destabilize rather than stabilize ionic transition states (see discussion of desolvation models in ref 36 and references given in this paper). In fact, the source of enzyme catalysis is the preorganization of a very polar environment.

Although the preorganization concept is uniquely defined, it is hard to assess it without actual calculations using the terms in eq 9 or 10. This problem can be illustrated by considering a recent work of Herschlag and co-workers,[246] who found that hydrogen bonding between ketosteroid isomerase and a series of transition-state analogues appeared to make only small contributions to the free energy of binding. The authors state that "there are many more water molecules in a volume of bulk solution to interact with a ligand than there are dipoles in the corresponding volume of an enzyme interior, and it remains unclear how precisely preoriented these dipoles are". Actually, the degree of preorganization of the dipoles and their electrostatic contribution can be quantified in a clear way if one uses a proper computational analysis (as is now accepted in the field of electron transfer modeling (e.g., ref 87)).

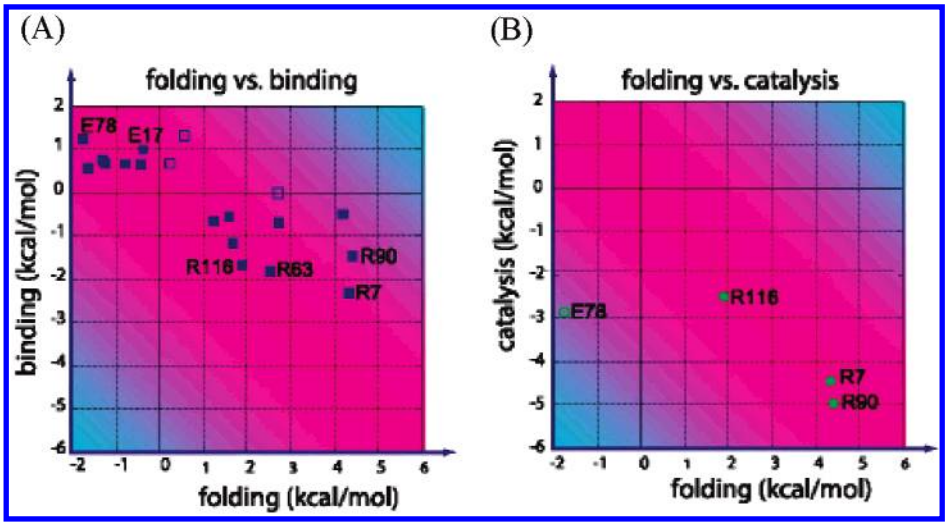


Figure 12. Demonstration of the anticorrelation between the energy contributions to folding and those (A) to binding or (B) to catalysis in CM. The blue and the red designate respectively the correlated and the anticorrelated regions. Each point represents the contribution from the designated residue of CM to folding and to binding (A) or to catalysis (B). Most of these points exist in the region of anticorrelation. The figure summarizes our preliminary results for the stability/activity contributions in CM. The calculations described here estimated the folding energy by the PDLD/S-LRA model and the formulation described in ref 106. The catalytic effect and the binding energy were evaluated by the microscopic LRA procedure with the EVB charge distributions of the RS and TS.

At this point, it may be useful to mention that Jencks's idea that enzymes use their binding energies to destabilize the substrate and to bring about the positioning of the reacting groups. Actually, a large part of the preorganization effect is due to the inherent folding energy and not due to the interaction with the substrate. Furthermore, the preorganization effect results in transition-state stabilization rather than ground-state destabilization. A recent experimental finding that is in major conflict with Jencks's proposal is discussed in section 5.6.

4.3. The Cost of Electrostatic Preorganization Is Paid by the Folding Energy

As stated above, it appears that the catalytic power of enzymes is largely due to the preorganized electrostatic environment of their active sites. Our considerations of the overall energetics of this effect led to the idea that the preorganization is associated with reduction in the protein folding energy.[1,101] This stability/activity idea was also supported by experimental works[102,103] and electrostatic modeling.[104] However, despite the importance of this issue, it has not been subjected to careful computational studies. To clarify this relationship, we performed preliminary studies that considered the catalytic reaction of CM and evaluated the contributions of the residues to binding, catalysis, and stability. The contributions to binding and catalysis were evaluated both by the semimacroscopic PDLD/S-LRA approach and by the microscopic LRA approach (see e.g. refs 8 and 105 for related calculations). The contributions to protein stability were evaluated by the PDLD/S-LRA method using the formulation outlined in ref 106 (see ref 107 for related formulations). Our preliminary results are summarized in Figure 12.

As seen from the figure, we obtained an interesting anticorrelation between the group contribution to folding and the group contribution to binding. As much as catalysis is concerned, we obtained similar but less pronounced anticorrelation. Note that, in the case of CM, the same preorganization effects that stabilize the TS also stabilize the RS (the RS and TS have similar charge distributions, as explained in section 5.4). We consider these preliminary results quite encouraging. Obviously, much more detailed studies, as well as comparison to mutation experiments, are essential, and such studies are now underway in our lab.

4.4. Metal Ion Catalyses

The importance of metal ions in enzyme catalysis has been emphasized by many workers (e.g., ref 108) and analyzed in specific cases.[109,110] In this work, we will consider the metal ion as a part of the enzyme environment, although consideration of the metal ion as a part of the reacting system is also possible. A typical effect of a metal ion is described in Figure 13, which describes the catalytic reaction of SNase.[111] Basically all the catalytic effect in this case can be attributed to the electrostatic interaction between the Ca^{2+} and the transition state. Furthermore, the changes in the catalytic effects as a result of substituting the Ca^{2+} ion by other metal ions have been reproduced in a semiquantitative way[110] in terms of the change of the electrostatic effect of the different metal ions (represented by the proper change in the ionic radius[110]).

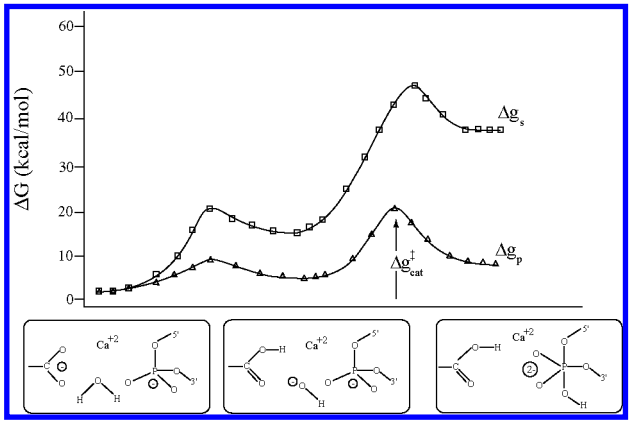


Figure 13. Calculated free energy profiles for the reaction of SNase and the corresponding reference solution reaction. (Reprinted with permission from ref 111. Copyright 1989 American Chemical Society.) The lower inset represents the reaction steps.

The same type of electrostatic stabilization has been found in cases of other metalloenzymes.[110,112−114] All these cases were modeled by describing the metal ion in terms of its electrostatic effect. This was done on different levels including a six-center model of the metal.[110] This type of force field reproduced both the observed solution and the solvent structure for the given metal ion in water. At any rate, EVB studies of metalloenzymes accounted for the observed effect of the metal in a semiquantitative way. Thus, we concluded that metal ions lead to a major catalytic effect, which is associated with their large electrostatic effects. It is also instructive to note here that the electrostatic effect of the metal is far from trivial. This effect is drastically different in water, in the gas phase, and in the enzyme site.

One may still try to argue that the effect of the metal ion can be considered as a covalent rather than an electrostatic effect. One should take into account similar considerations to those used in the discussion of a low-barrier hydrogen bond,[59] since we have to define clearly electrostatic and covalent interactions. Although an in-depth valence bond analysis of this issue is beyond the scope of the present work, we point out that the use of the same parameters that reproduce the observed solvation free energy for different metal ions has reproduced their catalytic effect, and thus, the catalytic effect of metalloenzymes can be categorized as an electrostatic effect. Furthermore, the use of such an electrostatic model has clear predictive power, making it a powerful structure−function correlator.

4.5. Some Comments on the Energetics of Zwitterionic Transition States

Some of the most effective modes of electrostatic stabilization involve the stabilization of ion pair (zwitterions) type transition states by the preorganized polar environment of the protein (see, e.g., ref 14). However, the requirement for stabilization of ionic TSs is not always clear. Thus, it is useful to mention here some recent discussions of this issue.

As will be mentioned in section 5.1, many desolvation proposals involve ionized residues in nonpolar environments. Such residues would be un-ionized in nonpolar sites. Moreover, in any specific case, when the structure of the active site is known, one finds by current electrostatic models a very polar (rather than nonpolar) active site environment near the chemically active part of the substrate. A case in point is pyruvate decarboxylase, which was put forward as a classical case of RSD by desolvation.[115] However, the structure of this enzyme[116] appeared to be very polar. Unfortunately, despite the obvious fact that groups near charges were in polar rather than nonpolar environments, it is still assumed by some (e.g., ref 117) that ion pairs are stabilized in nonpolar environments and that this is the way pyruvate decarboxylase catalyzes its reaction. However, as clarified in many of our papers, ion pairs are destabilized (relative to water) rather than stabilized.[118]

A more reasonable view of the energetics of zwitterionic transition states has been advanced recently by Richard and co-workers (e.g., ref 119). These workers pointed out the importance of having zwitterionic transition states and attributed correctly their stabilization to the protein polar groups. However, they suggested that the stabilization is due to having polar groups in a low dielectric that increases their effect. This view overlooks what has been learnt about protein dielectrics by consistent conceptual and theoretical studies (e.g., ref 120). That is, the dielectric constant in active sites or in heterogeneous environments cannot be described as having polar groups in a nonpolar environment; by this argument one can present water as a nonpolar environment with polar groups (see discussion in ref 120). Furthermore, the macroscopic dielectric constant (as defined by the dipole fluctuations) in active sites is quite large.[106,121] At any rate, we leave the discussion of the proper description of protein dielectrics to our previous extensive works (e.g., refs 75, 106, 120, and 121) and only mention here that a consistent discussion of the electrostatic catalysis of protein active sites can only be obtained by considering these sites as very polar and preoriented ones. The main point is that the energetics of ion pairs in proteins cannot be analyzed correctly by using macroscopic analysis and that the traps associated with other descriptions are discussed elsewhere (e.g., ref 106).

4.6. Allosteric Control of Catalytic Activity Is Also Associated with Electrostatic Effects

Allosteric effects control many enzymatic processes where interaction with another protein or with effectors drastically changes the catalytic activity of the given enzymes. A textbook case is, for example, the action of carbamoyl synthase. So far, all the systems that have been explored by consistent simulations are found to be controlled by electrostatic effects. We will consider below several prominent examples.

The activation of Ras by GAP provides a general example of a molecular switch that controls cell differentiation (e.g., see discussion in ref 8). Through our simulation studies of this system, we have shown that the binding of GAP leads to a major electrostatic stabilization of the TS for GTP hydrolysis both by the so-called arginine finger[122] and by

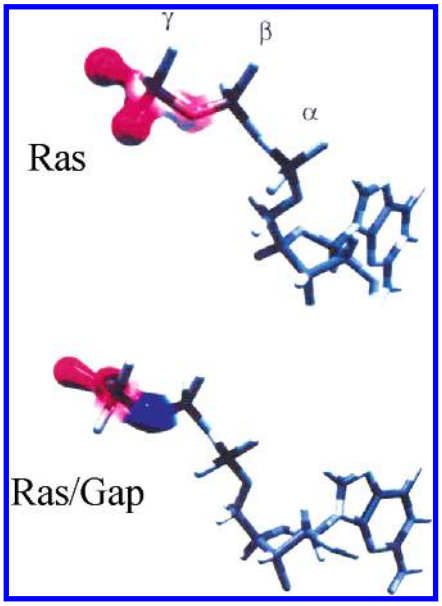


Figure 14. Change in electrostatic interactions, V_{qq}, between the protein residues and the γ- and β-phosphates of the substrate upon going from the reactant state to the transition state. This ΔV_{qq} is projected onto a surface around the phosphates for (a) Ras and (b) Ras/Gap. Blue indicates a stabilizing change in V_{qq} (the difference between the transition state and the reactant state is reduced), and red, a destabilizing change. The degree of stabilization or destabilization is proportional to the intensity of the color. Note that we are not presenting the change in electrostatic potential (which can be somewhat irrelevant), but rather the actual change in electrostatic energy. (Reprinted with permission from ref 112. Copyright 2000 American Chemical Society.)

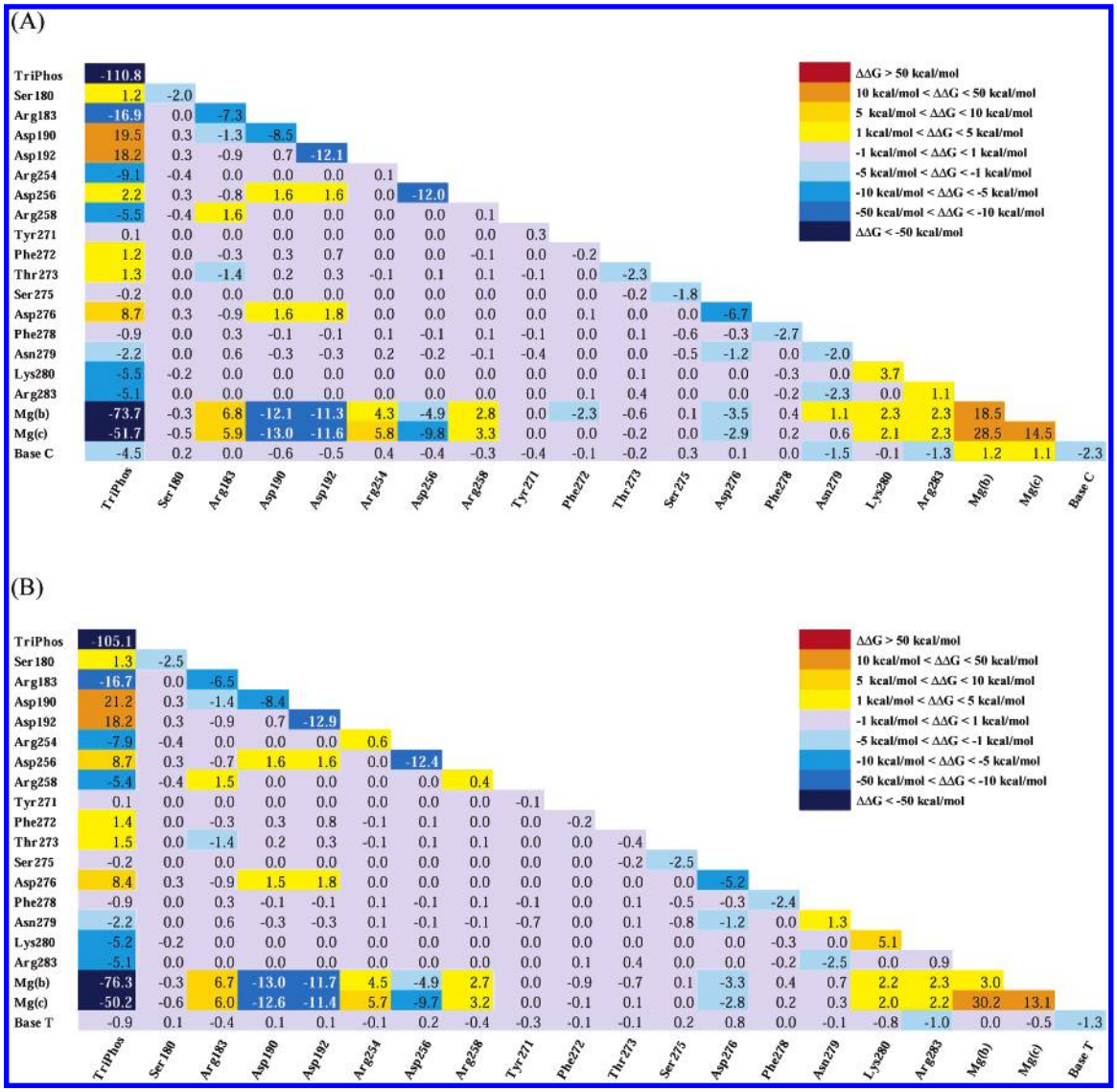


Figure 15. Interaction matrixes for the transition state of the incorporation of dNTP substrate in the active site of pol β calculated with the PDLD/S-LRA method. The diagonal elements describe the electrostatic contributions ($\Delta\Delta G_{ii}$ in kcal/mol) of the indicated residues to TS binding, while the off-diagonal elements describe the effect of the indicated *j*th residue (in the given column) on the TS binding by the *i*th residue ($\Delta\Delta G_{ij}$) (see eq 11). The intensity of colors corresponds to the strength of the interaction (e.g., red shows the strongest interaction; light gray, interactions close to 0; blue, negative interaction). (A) Template guanine with incoming dCTP ($\mathscr{R}$). (B) Template guanine with incoming dTTP ($\mathscr{Y}$). TriPhos denotes the triphosphate part of the incoming dNTP, Mg(b) denotes the binding magnesium ion, and Mg(c) denotes the catalytic magnesium ion. The bases are cytosine in part A and thymine in part B. (Reprinted with permission from ref 132. Copyright 2006 American Chemical Society.)

the transfer to a catalytic configuration, where the p-loop and other dipolar motifs stabilize the product of the hydrolysis reaction (see refs 8 and 112). This effect, which probably plays a general role in signal transduction, is illustrated in Figure 14.

The action of F_1-ATPase provides a general example of a molecular motor and a benchmark for simulation studies of energy transduction.[123−125] Our study of this system[126] has shown that the movement from the open to close conformation changes in a major way the stabilization of the TS for the ADP+Pi to ATP+water reaction.

The transition from the inactive chymotrypsinogen to the active chymotrypsin involves the cutting of the single bond between residues 15 and 16.[127] The new amino terminus at Ile-16 then forms a salt bridge with Asp 194, and this leads to a large shift of the main chain dipoles and the formation of the preorganized oxyanion hole.[128] The energetic of a related structural change due to the Gly-216/Gly-226 mutation to alanines was explored by EVB calculations[128] and shown to reflect changes in electrostatic TS stabilization.

Even in the case of hemoglobin, we were able to show[82] that a significant fraction of the allosteric effect is associated with the change in interaction between the charge shift upon oxygen binding and the change in protein tertiary structure (see discussion in ref 82).

The fidelity of DNA replication by DNA polymerases is controlled by the active site (where the incorporation reaction is catalyzed) and by the binding site of the incoming nucleotide that already includes the template base (e.g., see discussion in ref 113). The high fidelity is guaranteed by the fact that the rate of incorporation of an incoming wrong nucleotide, W, is drastically slower than the corresponding rate of incorporation of the right nucleotide, R (see ref 129). Now, the origin of this control can be quantified by considering the interplay between the binding site of the

incoming base and the stabilization of the TS in the chemical site. Our previous studies[130,131] already indicated that the binding of the incoming base is determined by the preorganization energy provided by the base binding site (that includes the template base); now the remaining challenge is to show that the TS stabilization by the preorganized active site is anticorrelated with the preorganization in the base binding site. This point has been explored in our preliminary studies, when we generated an "interaction matrix" to describe the interaction between the TS and the protein groups as well as the interaction of the base of the incoming nucleotide with its surrounding (Figure 15).[132] Using such diagrams for the R and W systems (at the corresponding relaxed TS structures) provides an instructive decomposition of the allosteric effect that controls replication fidelity. In particular, taking the difference between the R and W matrixes helps to identify the residues that are involved in the transfer of information from the base site to the transition-state site. Without going into the details (which will be addressed elsewhere), we note that the transfer of information between the base site and the chemical active site is controlled by electrostatic energies.

5. What About Other Proposals?

Although we have brought in compelling evidence for the overwhelming importance of electrostatic contributions, it is important to consider other proposals. This issue has been discussed extensively elsewhere (e.g., refs 1, 10, 27, 87, and 133), but it seems appropriate to summarize the results of computer modeling of the main alternative proposals.

5.1. Ground-State Destabilization by Steric Strain Does Not Provide a Large Catalytic Effect

The idea that enzyme catalysis is associated with ground-state destabilization was put forward in the classical studies of lysozyme.[134] Later studies that examined the actual amount of energy associated with steric strain found it to be small, due to the inherent flexibility of proteins.[1,40,135] Nevertheless, the strain proposal has been invoked in several recent studies, which will be considered below.[136,137]

Spectroscopic studies were interpreted as a ground-state destabilization due to electrostatic effects (electrostatic strain-induced mechanism). This idea was further elaborated recently by Anderson.[138] Unfortunately, the logic of ref 138 is problematic. That is, as already clarified by Warshel and Russell,[93] active sites that are designed to stabilize the transition state will an exert electric field on the ground states of related substrate analogues with chromophoric parts and lead to spectral changes. Such spectral shifts can clearly be used to establish the existence of the active site electric field and to quantify the fact that this field polarizes the ground state of the substrate. However, this polarization cannot (and should not) be used to establish substrate destabilization. More specifically, the main misunderstanding in refs 138 and 139 is the assumption that polarizing a substrate corresponds to its destabilization. In fact, applying a field that complements the ground-state charge distribution will stabilize the substrate by the product of the field and the dipole of the substrate, according to the expression

$$\Delta G_{sol}^{(1)} = -\zeta(\mu^0 + \Delta\mu) \tag{12}$$

where ζ is the local field and $\Delta\mu$ is the increase in the dipole due to electronic polarization. We also have to consider the penalty for the polarization energy ($-\zeta\Delta\mu$), which is approximately given by[93]

$$\Delta G_{sol}^{(2)} = +\frac{1}{2}\zeta\Delta\mu \tag{13}$$

(see ref 140 for related considerations).

Thus, the overall effect will be ground-state stabilization rather than destabilization. Of course, proper considerations must determine the direction of the ground-state permanent dipole. It is also important to consider the reference stabilization in solution.

At any rate, the main effect of the field from the preorganized active site is to stabilize the TS and not to destabilize the RS, and this fact has been established in many detailed computational studies that actually examined this issue (e.g., see Table 5).

Despite the overwhelming evidence that the strain hypothesis does not explain enzyme catalysis, it may be useful to consider the Co−C bond cleavage in coenzyme B_{12} enzymes. This system involves a radical bond breaking process and yet displays a very large catalytic effect of about 12 orders of magnitude.[141,142] This catalytic effect has been attributed to RSD and, in particular, to the distortion of the corrin ring or other strain effects.[84,91,141−145] In particular, it was suggested that the strain is operated by the so-called mechanochemical trigger mechanism associated with the upward folding of the corrin ring (e.g., refs 146−148). However, recent theoretical studies show that such a compression cannot destabilize the Co−C bond (e.g., refs 149 and 150). A recent QM/MM study[145] provides an impressive analysis of the system and reproduces the catalytic effects. The decomposition of the catalytic effect resulted in an about 8 kcal/mol electrostatic effect (between the protein and the leaving group) and an about 15 kcal/mol strain in the leaving group. However, decomposition to energy contributions in QM/MM calculations, that do not involve free energy calculations and sufficient sampling and relaxation (e.g., see ref 151), is extremely challenging and can lead to unstable results. A more recent study[152] that used the EVB method and very extensive free energy umbrella sampling calculations found, in agreement with ref 145, that the catalysis is due to the interaction with the leaving group, but the authors concluded that this effect is almost entirely an electrostatic effect (the catalysis disappears with a hypothetical, fully nonpolar leaving group). The study of ref 152 also used the LRA approach and established that the enzyme does not use RSD and stabilize the substrate more than water does. The enzyme stabilization of the leaving group increases, however, when the Co−C bond is stretched during the movement to the TS.

5.2. Dynamical Effects Do Not Contribute Significantly to Enzyme Catalysis

The proposal that special "dynamical" effects play a major role in enzyme catalysis (e.g., refs 153 and 154) has become quite popular in recent years (e.g., refs 155−163). To explore the validity of this proposal, it is essential to be clear about the definition of dynamical effects and to examine carefully whether the corresponding contributions are different in enzymes and in solution. Although this issue has been analyzed in great detail in several recent reviews,[17,87,164] we

will consider here some key points as well as some recent works that supported the dynamical proposal.

There are several ways to define dynamical effects, and these ways will be considered below. However, to provide the dynamical contribution to catalysis by a given definition, we must find different magnitudes of dynamical contributions to the rate constant in the enzyme and in water. Now, in considering different definitions, we may start with the transmission factor, since it is agreed in the chemical physics community (see references in ref 17) that all the dynamical effects are contained in this factor that corrects the absolute rate theory for recrossing of the reactive trajectories (see ref 87 for a clear definition). To the best of our knowledge, all the reported simulation studies going back to the earliest analysis[165] and to subsequent studies (e.g., ref 101) found that the transmission factors are similar in enzyme and in solution and do not differ much more than unity in the enzyme (e.g., refs 17 and 160).

Typical values of the transmission factors are 0.8 and 0.6 in enzyme and solution, respectively.[166] These values are too similar to each other to be considered as a source for any catalytic effect. Some workers[84,167] include the "nonequilibrium" effects in the transmission factor. However, it is not entirely clear as to what is meant by this. If, as seems to be implied by ref 167, the nonequilibrium term reflects nonequilibrium solvation, it seems to us (see below) that this effect does not belong in the preexponential term, since it is a well-defined contribution to the activation free energy. If instead the nonequilibrium term refers to some coherent motions, it is entirely unclear that there are current evidences or computational treatments that can explore such effects in condensed phases. Thus, we prefer to follow the eloquent discussion given in ref 168 and to keep only recrossing effects in the transmission factors (quantum tunneling effects are best assigned to $\Delta g^{\#}$ as a probability factor (see, e.g., refs 169 and 170).

Another definition can imply that dynamical effects are related to the availability of special coherent motions. In this way, the dynamical proposal implies that enzymes "activate" a special type of coherent motions, which are not available in the solution reaction. Now, the difference between the reaction in enzyme and in solution cannot be accounted for by evaluating the corresponding $\Delta g^{\ddagger}$ using nondynamical Monte Carlo (MC) methods. In other words, if the results from MC and MD are identical, then we do not have dynamical contributions to catalysis. Careful and systematic studies (e.g., refs 17 and 171) have shown that the reactions in both enzymes and solutions involved large electrostatic fluctuations. However, these fluctuations follow the Boltzmann distribution and, thus, do not provide dynamical contributions to catalysis.

It has been suggested (e.g., ref 160) that dynamical effects are associated with the so-called nonequilibrium solvation effects, which have been shown to be very problematic (see refs 17 and 87). Furthermore, it has been clearly demonstrated that the difference between the nonequilibrium solvation effects in enzyme and that in solution is an integral part of the difference between the corresponding activation barriers.

Apparently, there is no single experimental finding that can be used to consistently support the dynamical hypothesis. Most of the experiments that were used to support this proposal have not compared the catalyzed and uncatalyzed reactions and, thus, have not addressed the issue of catalysis (see discussion in ref 17). Instructive NMR experiments (e.g., ref 161) demonstrated the involvement of different motions in enzymatic reactions (see also below). The obvious existence of motions that have components along the reaction coordinate does not constitute a dynamical effect unless these motions are shown to be coherent. Probably, all the motional effects identified so far are related to entropic factors (i.e., to change in the available configurational space) rather than to real dynamical effects.

At this point, we find it useful, despite our previous reviews of the dynamical proposal (e.g., ref 164), to consider the most recent work that implied or explicitly supported this idea. We start by recognizing that the advance in NMR studies (e.g., refs 161 and 172) allows one to probe the interesting nature of the relatively slow protein motions. This, however, does not prove that proteins can "harness thermal motions through specific dynamic networks to enable molecular function" as suggested by ref 172.

An instructive example of what we see as an overinterpretation of exciting experimental findings is a recent follow up[173] to the study of ref 161. That is, study of the action of cyclophilin,[161] found that the protein motions are correlated with the substrate turnover. Now, the more recent study of ref 173 found that the same motions still exist in the absence of the substrate. This led to the interesting proposal that both protein structure and dynamics have coevolved synergistically and that dynamical presampling is "harvested for catalytic turnover". Unfortunately, while the findings of ref 173 are interesting, the analysis of the catalytic effect is far from conclusive. First, the authors do not address the facts that catalysis must be defined relative to a reference reaction in solution and that the catalytic effect of virtually every enzyme that has been studied consistently has been found to be associated with electrostatic rather than dynamical effects (this is true also in the present case, e.g., ref 174). Second, motions between two configurations that are involved in a reaction cannot contribute to catalysis if they occur relatively slowly in the absence of the substrate, because their slow rate implies a preexisting barrier for the reaction. A truly catalytic enzyme should push the free energy minima of the reactant and product states close together along the reaction coordinate (in the isolated enzyme surface) in order to minimize the reorganization energy.

A recent theoretical work[175] that was considered as a support of the finding of ref 173 has attempted to evaluate the dynamical contribution from the protein vibrations to the transmission factor of the erection of cyclophilin, and the authors of this report concluded that the dynamical contribution is significant. This study propagated trajectories from the TS, placing different amounts of kinetic energy in the protein normal modes. Unfortunately, this work involved major problems. First, adding arbitrarily non-Boltzmann energy to specific modes at the transition state, or any other state, has no relationship to correct rate theories. One has to prove that these vibrations are populated in a non-Boltzmann way and then to use a correct density matrix or an alternative treatment to examine if there is any validity to such an assumption. In other words, adding arbitrary kinetic energy in the direction of the product will certainly change the recrossing in any model and, thus, cannot serve as a way of examining the contributions of the protein mode; this challenging problem can perhaps be address by starting an assumed coherent mode from the ground state and examining if the coherence is retained in the long time that it takes to

reach the TS. Second, the same approach, whether justified or not, should have been performed on the reference solution reaction. Such a study would almost certainly reproduce a similar effecting solution and thus correspond to little or no catalytic effect.

Another recent theoretical attempt to support the dynamical proposal[176] used transition path sampling to explore the catalytic reaction of lactate dehydrogenase (LDH). It was concluded that some trajectories in the TS region move in a concerted way and that some move in a stepwise path, and this was used to imply that the enzyme dynamics helps to catalyze the reaction. However, this study also involved several problems. First, no attempt has been made to evaluate the activation free energy and no comparison has been made to the uncatalyzed reaction, in contrast to earlier studies that actually elucidated the role of the reduction in reorganization energy in the same enzyme.[177] Second, the fact that the reaction path may involve both concerted and stepwise paths has little to do with dynamical effects. It simply reflects the shape of the calculated reaction surface. It may also be useful to point out that the transition path sampling approach may have been useful in exploring the activation free energy, but the nature of the productive trajectories could be easily explored by running downhill trajectories, as done in many other studies (see ref 164).

Other studies that emphasized the correlation between the protein motions rather than dynamical effects per se will be considered in section 5.3.

To summarize this discussion, it is useful to recognize that consistent simulation studies found no evidence for dynamical contributions to catalysis.

Another related issue is associated with the suggestion that vibrationally enhanced tunneling (VET) plays a major role in enzyme catalysis (see, e.g., refs 162 and 163). Some workers (e.g., ref 163) assumed that there exists here an entirely new phenomenon that makes TST inapplicable to enzymatic reactions. However, the VET effect is not new and is common to many chemical reactions in solution.[179−181] Moreover, the VET is strongly related to the transition state theory (TST). That is, when the solvent fluctuates and changes the energy gap (see refs 171 and 179), the light atom sees a fluctuating barrier that allows in some cases for a larger rate of tunneling. As shown in ref 171, these fluctuations are taken into account in the statistical factor of the classical TST and the same is true when quantum effects are taken into account. Thus, the recent finding that the solvent coordinates should be considered in tunneling studies is not new and does not mean that this effect is important in catalysis.

Hwang et al. were the first to calculate the contribution of tunneling and other nuclear quantum effects to enzyme catalysis.[182] Since then and in particular in the past few years, there has been a significant increase in simulations of quantum mechanical−nuclear effects in enzyme reactions. The approaches used range from the quantized classical path (QCP) (e.g., refs 17, 183, and 184), to the centroid path integral approach,[169,170] to vibrational transition-state theory,[185] to the molecular dynamic with quantum transition (MDQT) surface hopping method.[186] Most studies did not yet examine the reference water reaction and, thus, could only evaluate the quantum mechanical contribution to the enzyme rate constant, rather than the corresponding catalytic effect. However, studies that explored the actual catalytic contributions (e.g., refs 17, 164, 183, and 184) concluded that the quantum mechanical contributions are similar for the reactions in the enzyme and in solution and, thus, do not contribute to catalysis.

5.3. Correlated Modes Clearly Exist in Proteins, but They Also Exist in Solution

Early studies of Benkovic, Wright, and co-workers[158,187,188] have studied the reaction of dihydrofolate reductase with NMR. They found that site-directed mutations of the residues in a loop that undergoes relatively large backbone motions had detrimental effects on catalysis, and they suggested that the dynamics of these residues could be important for catalysis. This suggestion was supported by Brooks and co-workers,[157,189] who carried out MD simulations of three ternary complexes of the enzyme. However, these studies did not examine any of the transition states in the reaction or demonstrate any dynamical effects on the rate constant.

More recent studies (e.g., refs 190−192) have led to growing recognition that the mutational effects in DHFR reflect equilibrium structural effects rather than dynamical effects. However, the focus has shifted to discussion of correlated motions (e.g., refs 193 and 194) rather than of the reorganization effects considered in Figure 11. This seems to create an impression that here we have a special catalytic effect with new implications beyond the concept of electrostatic transition-state stabilization. However, the identification of correlated motions does not provide a new view of enzyme catalysis, because reorganization of the solvent along the reaction path in solution also involves highly correlated motions.[171,182] Correlated motions of an enzyme do not necessarily contribute to catalysis and, indeed, could be detrimental if they increase the reorganization energy of the reaction. Our EVB and dispersed-polaron approaches described elsewhere (e.g., ref 87) consider the enzyme reorganization explicitly and automatically assess the complete structural changes along the reaction coordinates. A dispersed-polaron analysis of the type represented in ref 87, for example, determines the projection of the protein motion on the reaction coordinate and provides a basis for a quantitative comparison with a reference reaction in solution. In other words, our studies indicated quite early that the motions along the reaction coordinate involve many modes in both the enzyme and solution reactions, but we could not find any evidence that the existence of coupled modes contributes to catalysis.

One may still wonder about the connection between correlated motions and the effect of mutations on enzyme catalysis. However, the effect of distant mutations in DHFR is likely to be due to propagation of structural changes to the active site region, as is the case in many allosteric systems (e.g., refs 82 and 112). The new active site configuration is then unable to provide the same preorganized environment as the native enzyme. In other words, the mutation can change the curvature of the reaction coordinate and this change can be described as the effect of coupled modes (although such a description is neither predictive nor particularly useful). However, the issue is not the decomposition of the reaction path to the different protein modes but the height of the activation barrier. This barrier is determined by the reorganization energy, which depends on the sum of the displacements of the different modes upon motion from the reactant to the product state. Apparently, the mutations lead to an increase in the distance between the product and reactant states and in fact to larger displacements of the

modes that are projected on the reaction coordinate. This means that the coupled modes reduce rather than increase the catalytic effect.

Perhaps the most effective way to classify and quantify the effect of mutation energy is to use allosteric diagrams of the type discussed in ref 132 and section 4.6. In this case, the focus is on the transfer of information due to energy coupling rather than just the correlation between simulated structural changes, and the relationship to the active site preorganization is clearer.

5.4. Near Attack Conformations (NACs) Correspond to TS Stabilization

Bruice and co-workers have advanced the idea that enzymes catalyze reactions by favoring configurations in which the reactants are pushed to a close interaction distance (e.g., ref 88). In most cases that we have studied, the energy associated with moving the reacting fragments from their average configuration in water to the average configuration in the enzymes was small, indicating that the corresponding catalytic effect was relatively minor.[96,195] In one case, where the NAC effect appeared to be large, it was found that the actual catalytic effect was attributable to electrostatic stabilization of the transition state.[90] In other words, the NAC effect evidently has been found to be a consequence rather than the reason for the electrostatic catalytic effect.[90]

The most notable example is chorismate mutase (CM), whose RS and TS are illustrated in Figure 16. As discussed in ref 90, both the RS and TS of CM have similar charge distributions, and thus, the same preorganization effects that stabilize the RS also stabilize the TS and lead to an apparent NAC effect by making the RS structure closer to that of the TS. However, this is an automatic result of the TS stabilization rather than being the reason for catalysis (see also the caption of Figure 16).

5.5. The Entropy Contributions of Bringing the Reactants Together Do Not Lead to a Large Catalytic Effect

The idea that enzyme catalysis is associated with the entropy loss upon substrate binding was advanced in the early work of Jencks and co-workers[60,196] and has gained some support in recent computational studies.[197,198] However, Villà et al. have shown that this proposal is based on an incomplete thermodynamic cycle.[18] The entropic contribution probably cannot be large since the activation entropy in solution is usually much smaller than one might assume. This reflects the fact that the formation of the transition state does not lead to loss of many degrees of freedom.[18] Problems with the entropic proposal also have emerged from experimental studies of cytidine deaminase by Wolfenden and co-workers.[199]

5.6. Reactant State Destabilization by Desolvation Effects Does Not Provide a Large Catalytic Effect

The idea that enzymes reduce the activation barrier by desolvating and destabilizing the ground states of their reacting fragments has been put forward by many workers (e.g., refs 60, 83, 200, and 201). However, systematic analyses have demonstrated that the TS is solvated much more strongly in many enzymes than in the reference solution system.[1,27,95] It is important to note that the only way to test the desolvation proposal computationally is to calculate the actual binding energies of the reactants in the ground and transition states (see, e.g., ref 95). Most of the computational studies that are claimed to favor the desolvation proposal have not included such calculations.

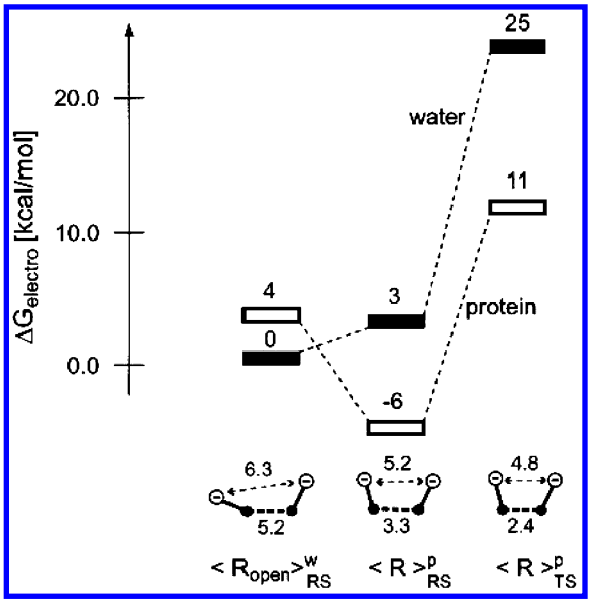


Figure 16. LRA estimates of the electrostatic energy for several points along the reaction coordinate. Energies in kilocalories per mole are indicated over the corresponding bars. Distances in angstroms are given for the separation between the carboxylate charge centers (designated by (−)) and for the distances between C_1 and C_9. The relative positions of the protein and water profiles are set in a way that the binding energy at $\langle R \rangle^{p}_{RS}$ will correspond approximately to the observed ΔG_{bind} (−5.6 kcal/mol). The TS free energy in water includes a constant term that reproduces the corresponding observed value (since the LRA electrostatic contribution does not include the intermolecular activation energy). The same constant is used for the protein TS. (Reprinted with permission from ref 90. Copyright 2003 American Chemical Society.) The figure illustrates that the reduction in this RS energy at $\langle R \rangle^{p}_{RS}$, relative to the corresponding energy in water, and the fact that, in water, the energy at $\langle R \rangle^{p}_{RS}$ is higher than that in $\langle R \rangle^{w}_{RS}$ simply reflect the TS stabilization effect. That is, the protein field that stabilizes the TS charges by 14 kcal/mol also stabilizes the RS charges (since the charge distribution is similar in the TS and RS).

One of the best illustrations of the problem with the RSD proposal has been given in the case of orotidine 5′-monophosphate decarboxylase (ODCase).[36] Although this case was discussed extensively, it gained an additional importance due to a recent experiment[202] that justifies taking this as specific general example. Now, the catalytic action of ODCase was first proposed to involve the desolvation effect.[201] This was shown to involve an incorrect thermodynamic cycle (e.g., ref 36). The elucidation of the structure of this enzyme showed that its active site is extremely polar (highly charged), but this led to a new RSD proposal where the negatively charged groups of the protein destabilize the carboxylate of the orotate substrate.[50] This proposal was shown to be inconsistent with the nature of the system, since a destabilized orotate will accept a proton and become stable.[36] Furthermore, careful computational study illustrates that the protein works by TSS and not by RSD (see ref 36 and discussion below). Finally, recent studies by Wolfenden and co-workers[203,204] have provided strong evidence against the RSD proposal. These studies demonstrated that mutations of Asp96 and other residues that were supposed to destabilize the orotate led to weaker rather than stronger binding. As predicted in ref 36, this result is inconsistent with the RSD,

since destabilization of the RS should result in a reduction of the binding energy.

All the above points were already discussed and analyzed extensively (e.g., ref 27), but the new experiment of Amyes et al.[202] illuminates the problem in a new light. These workers reexplored the origin of the catalytic power of ODCase by studying the decarboxylation of a truncated substrate (called EO) that lacks the 5′-phosphodianion part. They found that while the reaction of this substrate is quite slow, the binding of exogenous phosphate dianion to ODCase results in a 80000-fold increase in k_{cat}/K_m. This appeared to be in clear conflict with the proposal that the presumed RSD is due to the binding free energy of the 5′-phosphodianion part of the substrate, which is supposed to induce extremely large reactant RSD and thus to catalyze the reaction (e.g., ref 50). In this proposal the negatively charged groups of the protein are used to destabilize the carboxylate of the orotate. In fact, this view has been used as a confirmation of Jencks's proposal that enzymes work by using binding energies to destabilize the ground state of the reactive part of the substrate.

However, the new work of Amyes et al.[202] indicates that the RSD idea is incorrect and, thus, confirms a careful analysis of this issue by Warshel et al.[36] That is, as pointed out in ref 50, the GSD requires that the phosphate part will be bound so strongly that it would pull the chemical part to its destabilizing environment. Unfortunately, the experiment of Amyes et al.[202] shows that the catalysis occurs in the absence of a bond between the phosphate and the EO parts, so that the presumed strain cannot be transferred between these parts. Of course, the binding of the negative part of the substrate does help the active site to reach its proper preorganization and, thus, to use it for electrostatic stabilization of the TS. This, however, has little to do with the classical idea of using binding energy for RSD.

Despite the above experimental and theoretical demonstration of the problems associated with the RSD proposal in the case of ODCase, some studies are still interpreted in terms of the likeliness of this proposal. Thus, it is useful to consider several of the seemingly strong arguments put forward against the TSS proposal in a recent review.[91] The first point is related to the observation that the FEP bonding calculations of Wu et al.[50] that serve as a basis for the RSD proposal did not reproduce the catalytic effect that was reproduced by the PMF calculations by the same research group. Having two very different results from seemingly reliable calculations that supposedly explain a given experimental fact indicates that one of them is problematic. This discrepancy was explained by the argument[91] that FEP binding calculations of the RS and TS do not reflect the effect of the protein reorganization, and basically correspond to fixed protein configurations. Unfortunately, this argument overlooks the fact that correct free energy calculations must reflect all effects including certainly the protein reorganization and, of course, complete the same overall thermodynamic cycle. The fact that FEP and PMF (umbrella sampling) approaches must obey the same thermodynamic laws has also been illustrated in a recent work.[205] In fact, the problem with the idea that FEP calculations do not reflect the reorganization energy can easily be established by evaluating the free energy of a charge in water, where half of the FEP result is associated with the solvent reorganization. The fundamental problem with the argument of ref 91, as well as the resulting concept of catalysis by conformational changes, will be further considered at the end of this section.

The second point brought up in ref 91 involves the claim that the theoretical model of ref 36 that took the (orotate + Lys 72) as the reaction region is problematic (since Lys 72 is a part of the protein), and thus, presumably this model cannot be used to support the TSS idea. However, Warshel et al.[36] also obtained the same TSS results regardless of whether they included Lys 72 in the reaction region or the surroundings (this is, of course, a requirement for any correct calculation). It is just simpler to explain the catalytic effect if one considers the Lys as a part of the reacting region. Furthermore, including Lys 72 in the reaction center is as valid as including His 57 in the calculations of serine proteases, and using His 57 as part of the reaction center is a key element for any correct treatment of these enzymes. Finally, Gao et al. argued[91] that the treatment of the [orotate$^-$−Lys$^+$] as an ion pair is incorrect, since presumably increasing the ion pair distance from 4 to 6 Å (see Figure 3 in ref 91) will increase the energy of the system by 28 kcal/mol. Here again, it is important to put the discussion in terms of proper electrostatic concepts, before questioning consistent energy treatments. That is, the fact that the ion pair distance increases upon going to the TS has been established in the ab initio calculations of ref 36, and it is also a fact that the increase in energy in this process is smaller in the protein than in water. The energy goes down in this process relative to the case in water. Apparently, ref 91 overlooked the fact that the same 28 kcal/mol *gas-phase* energy increase also occurs in the reference solution reaction. Furthermore, it also overlooked the fact that the 28 kcal/mol is almost completely compensated for by the increase in solvation and it is really around 1−3 kcal/mol (e.g., see Figure 16 in ref 93). Now what the protein preorganized polar environment does is to stabilize the ion pair much more than water does.[14,17] Although this point cannot be reproduced by the dielectric models considered in ref 91, it can be quantitatively reproduced by both microscopic and semimacroscopic electrostatic calculations. Here again, it is important to emphasize the crucial importance of understanding the energetics of ion pairs in proteins when exploring different hypotheses about enzyme catalysis.

It might be useful to address at this point the attempts[84,91] to formulate the presumed RSD as "catalysis by enzyme conformational changes", proposing that the enzyme is pushed toward an unstable structure in the reactant state and then it relaxes to a lower energy structure at the TS. Unfortunately, this proposal has not been supported by consistent calculations. That is, the actual change in the protein internal energy, ΔG_{pp} (in the notation of ref 91), can only be estimated at present by proper calculations of reorganization energy using either the Marcus parabola or by the LRA treatment (e.g., eq 10b). Instead, the existence of a large negative contribution from $\Delta\Delta G^{\ddagger}_{pp}$ was inferred from the inconsistent assumption that the FEP difference in binding energies of the TS and RS (designated here by $\Delta\Delta G^{FEP}_{bind}$) cannot reproduce the correct activation free energy, since presumably the FEP results do not include the reorganization energy.

Thus, it was assumed that we can use the relationship

$$\Delta\Delta G^{\ddagger}_{pp} = \Delta\Delta G^{\ddagger} - (\Delta G_{bind}(TS,r_{TS}) - \Delta G_{bind}(RS,r_{RS})) = \Delta\Delta G^{\ddagger} - \Delta\Delta G^{FEP}_{bind} \quad (14)$$

However, as discussed above, $\Delta\Delta G^{FEP}_{bind}$ includes the reorganization energy and, when evaluated correctly, it is

equal to $\Delta\Delta G^{\ddagger}$, and the assumption that $\Delta\Delta G^{\ddagger}_{pp} = \Delta\Delta G^{\ddagger} - \Delta\Delta G^{FEP}_{bind}$ is not justified. Probably the only way to get eq 14 to work is to use a very large constraint on the protein, in the TS and RS calculations, but such a treatment is very problematic.

Basically, the catalysis is due to the pre-reorganization of the active site as proposed and quantified in refs 36 and 206 where the reorganization energy in the enzyme is positive but smaller than that in the water reaction. As to the $\Delta\Delta G^{\ddagger}_{pp}$ estimated in ref 91, it can be estimated correctly as a part of the reorganization energy (we say "a part", since the full reorganization energy is associated with going to the product state). Now, the reorganization energy in the case of ODCase is positive, since it compensates for the increase in the protein−substrate electrostatic interaction (upon moving to the TS). Perhaps the main misunderstanding here stems from the fact that ref 91 has not considered both the protein and water by the same formulation and does not evaluate the reorganization in water.

5.7. A Consistently Defined Low-Barrier Hydrogen Bond (LBHB) Proposal Leads to Anticatalytic Effects

It has been proposed that some enzymes catalyze their reactions by forming so-called low-barrier hydrogen bonds (LBHBs) with charged transition states.[178,207−209] The only significant distinction between this suggestion and the idea that preorganized hydrogen bonds stabilize the TS in the enzyme electrostatically[1] is that an LBHB is a partially covalent (delocalized) bond such as a bond of the form $Y^{-\delta}\cdots H\cdots X^{-\delta}$ where X^- can be, for example, a negatively charged oxygen atom of the solute in the TS (another example is given in Figure 17). Warshel and Papazyan[59] showed that an LBHB would lead to a reduction rather than an increase in the solvation of the TS and, thus, would have an anticatalytic effect. Enzymes appear to do a better job in stabilizing the TS with localized charges rather than with delocalized charges.[59] It is important to realize that gas-phase calculations that were used to support the LBHB proposal (e.g. ref 208) are irrelevant to enzyme active sites. All the current EVB studies (see discussion in ref 211 and the molecular orbital QM/MM studies that reach a sufficiently quantitative level[212−214] have contradicted the LBHB idea.

It might be useful to point out at this stage that, in contrast to some implications (e.g., ref 215), our considerations of the LBHB[159,216] are based on the EVB method, which is probably the best current approach to analyze the effect of the environment on covalent and charge-transfer effects in hydrogen bonding. That is, the EVB diabatic states and the covalent mixing terms are calibrated by forcing them to reproduce the ab initio ground-state surface and the changes in the charge distribution during the reactions in the gas phase and in solution. Adding the environmental effect to the energies of the diabatic state allows one to accurately examine the effect of changes in the environment on the charge transfer (CT) character of the given HB. This approach has been used in quantitative studies of assumed HBs to the environment[59,211] and demonstrated that the LBHB proposal cannot account for the catalytic effect of pre-organized HBs.

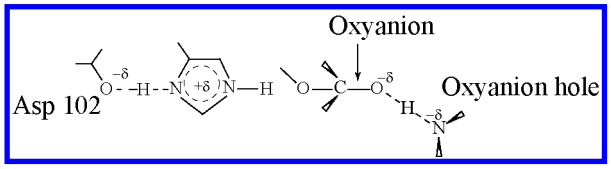


Figure 17. Illustration of the LBHB proposal for serine proteases. The figure demonstrates a valid covalent proposal (that might or might not be correct), where the active site groups (Asp 102 and the oxy-anion hole) form a partial covalent bond to the TS of the reacting system.

5.8. A Consistently Defined Covalent Catalysis Does Not Account for Large Catalytic Effects

The idea that enzyme catalysis resembles heterogeneous catalysis and the difficulty to quantify the energetics of enzymatic reactions have led to the proposal that enzymes catalyze reactions by covalent catalysis.[6] This idea was formulated in a book[6] that involved discussion of many enzymatic reactions but, unfortunately, no assessment of actual catalytic effects. Basically, it was argued[6] that "it has become more accurate, however, to say that catalyst alters the (uncatalyzed) chemical pathway of a reaction to one with a lower activation energy". This statement overlooks the consideration of section 2 and the fact that, if this were the actual effect of the enzyme, we would also have a reaction with the same low activation barrier in solution (this is our reference state). A similar support to the covalent idea has recently been advanced by Zhang and Houk,[5,217] who argued that a major part of the catalytic power of enzymes should be due to covalent effects, since the environmental effects cannot be more than the maximum binding energy, which they estimated to be around 15 kcal/mol. This assertion involves several major problems: First, as explained above, the origin of the catalytic effect is different from the origin of the binding of the substrate (defined here as the reactant state (RS) binding). Second, the factors assigned in ref 5 as covalent effects are the well-known effect of not having the same mechanism in the enzyme and in the reference solution reaction, and thus, they have very little to do with the real problem. Finally, as shown in section 2, we have clear cases with environmental contributions, which are much larger than the 15 kcal/mol assigned arbitrarily as the upper limit of environmental effects.

At this point, it is instructive to consider the assertion that the existence of different mechanisms in enzyme and solution is qualified as covalent catalysis. The issue is how the enzyme catalyzes its given reaction relative to the same reaction in solution. Once we recognize this fact, we can ask whether the very large environmental effect of the enzyme is associated with a covalent bonding between the active site and the TS of our well-defined reaction. In doing so, we must realize that the nature of the TS in, for example, general base catalysis has very little to do with the partial bond formation to the TS.[5] This is simply the bonding within the TS (which, of course, occurs both in the reference solution reaction and in the enzyme). A well-known example of a legitimate covalent proposal is the low-barrier hydrogen bond (LBHB) proposal (Figure 17), which explicitly assumes a partial covalent bonding between the TS and enzyme hydrogen bonding donors.

The interest in the covalent proposal reflects difficulties to rationalize a large environmental stabilization (this is a part of the rationale of ref 5), but the corresponding proposal is not useful unless it is stated correctly and explicitly, and thus can be actually analyzed. At present, any consistent

computational and conceptual attempt to examine the covalent proposal has resulted in the finding that the corresponding catalytic effect is either very small or nonexistent. Basically, it was found that the enzyme environment provides more stabilization to the localized TS charges than to the delocalized charges of the partially covalent arrangement.[59] Of course, one should examine seriously new specific covalent proposals, but no such proposal has been put forward in ref 5 nor analyzed in that work.

6. Problems with the Catalytic Antibody Proposals Reflect Difficulties with Creating a Proper Preorganized Environment

At this point in the review, it is useful to discuss the lesson from the field of catalytic antibodies. That is, studies of catalytic antibodies played a prominent role in the realization that enzymes stabilize transition states, since the antibodies were raised against haptens that were considered to be TS analogues.[218−221] However, because the catalytic power of such antibodies is usually much smaller than that of natural enzymes, some workers have concluded that TS stabilization cannot account for the full catalytic power of enzymes, and it has been suggested that the antibodies have less dynamical power than enzymes.[156] In one of the few computational studies that have addressed this point, the charge distribution in the TS of the reaction catalyzed by chorismate mutase was found to be quite different from that in the TS analogue used to elicit a catalytic antibody for the same reaction.[222] In many cases, it is not surprising that the catalytic antibody would be less effective than the enzyme, since the enzymatic reaction involves several transition states with similar energies and a *single* hapten cannot mimic the charge distribution in more than one of these states.[222] Related considerations with regard to the difficulties of preparing perfect transition-state analogues were eloquently presented in a recent work of Schramm.[223]

7. Conclusions

This review examines the nature and origin of the enormous power of enzymes. We started by defining and quantifying the catalytic effect. This was done by clarifying the importance of defining a proper reference state. In doing so, we defined a "chemistry-free" reference state that involves the same mechanism in the corresponding enzymatic reaction. This reference state eliminates the confusion caused by the fact that many enzymatic reactions involve different mechanisms from those that occur in the corresponding solution reaction. Moreover, it clarifies the fact that the energy associated with the change between the different mechanisms in solution can be easily evaluated, and thus, it has never been a part of the real puzzle of enzyme catalysis. The new catalytic scale also makes it clear that the enzyme environmental effect is extremely large and, thus, establishes the challenge of quantifying this environmental effect.

To clarify the nature of the electrostatic contribution to enzyme catalysis, we described and quantified the preorganization concept, demonstrating that enzyme active sites provide a preorganized polar environment that stabilizes the transition state much more than the corresponding environment in water. We also clarified and demonstrated that the preorganization effect involves a reduction in the folding energy and results in an inversed activity/stability correlation.

To establish the proposal that the catalytic effect is primarily due to electrostatic effects, it is important to demonstrate that the contributions from other factors and proposals are relatively small. This was done in the present review by considering various proposals and summarizing studies that established the problems with those proposals. Thus, although it is reasonable to assume that evolution has exploited many possible catalytic effects, it appears that, with the exception of the electrostatic preorganization effects, most of the mechanisms that have been proposed do not lead to significant catalytic effects. Of course, our findings cannot be extrapolated to enzymes that have not yet been studied. But the only way to examine the feasibility of a proposed effect is to assess its magnitude in a variety of known enzymes, and the finding that a particular effect is relatively unimportant in all of these test cases indicates that this effect cannot contribute significantly to catalysis.

It is important to comment here on the possible perception that our attempts to analyze different catalytic proposals involve a polemic and unnecessary focus on the problems with other studies. We feel that the only way to progress in this field is to use a clear energy-based analysis and to insist that this analysis will satisfy the laws of physics and chemistry. In doing so, it is crucial to clarify what has been meant by different proposals (e.g. dynamics, desolvation, RSD, entropic catalysis, etc.). The insistence on clear definitions and unique formulations is probably the best way to move from a state where all proposals are equally valid to a situation where some proposals can be eliminated, and this review is in some respect an attempt to encourage the readers to apply logical considerations (regardless of whether they agree with our perspective or not).

In summary, the present study and related works have provided a clear support to the view that electrostatic TSS is the most important factor in enzyme catalysis.[14,54] It also appears that the issue in studies of enzyme catalysis is not the reformulation of transition-state theory but the ability to evaluate the activation free energy in a reliable way, including, if needed, quantum corrections. We believe that the accelerated increase in theoretical studies will provide growing support to the electrostatic proposal and that the ability of such theoretical studies to reproduce experimental observations will lend credibility to their ability to dissect the overall catalytic effects to their key components and thus to establish the origin of enzyme catalysis.

8. Acknowledgments

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