PROTEINS
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REVIEW

At the dawn of the 21st century: Is dynamics the missing link for understanding enzyme catalysis?

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ABSTRACT

Enzymes play a key role in almost all biological processes, accelerating a variety of metabolic reactions as well as controlling energy transduction, the transcription, and translation of genetic information, and signaling. They possess the remarkable capacity to accelerate reactions by many orders of magnitude compared to their uncatalyzed counterparts, making feasible crucial processes that would otherwise not occur on biologically relevant timescales. Thus, there is broad interest in understanding the catalytic power of enzymes on a molecular level. Several proposals have been put forward to try to explain this phenomenon, and one that has rapidly gained momentum in recent years is the idea that enzyme dynamics somehow contributes to catalysis. This review examines the dynamical proposal in a critical way, considering basically all reasonable definitions, including (but not limited to) such proposed effects as "coupling between conformational and chemical motions," "landscape searches" and "entropy funnels." It is shown that none of these proposed effects have been experimentally demonstrated to contribute to catalysis, nor are they supported by consistent theoretical studies. On the other hand, it is clarified that careful simulation studies have excluded most (if not all) dynamical proposals. This review places significant emphasis on clarifying the role of logical definitions of different catalytic proposals, and on the need for a clear formulation in terms of the assumed potential surface and reaction coordinate. Finally, it is pointed out that electrostatic preorganization actually accounts for the observed catalytic effects of enzymes, through the corresponding changes in the activation free energies.

Proteins 2010; 78:1339–1375.

Key words: dynamics; enzyme catalysis; catalytic landscape; tunneling; promoting motions; single molecule experiments; coherence effects; polar preorganization.

INTRODUCTION

Enzymes play fundamental roles in almost all life processes. They accelerate a great variety of metabolic reactions and they control signaling, energy transduction, and the transcription and translation of genetic information. Their ability to catalyze reactions by many orders of magnitude allows cells to carry out reactions that otherwise would not occur on biologically relevant timescales. There is, therefore, broad interest in understanding the origin of this catalytic power on a molecular level.

Although many proposals have been put forward to rationalize the catalytic power of enzymes (see the special issue edited by Schramm in *Chem Rev* 2006 for a partial list),[1] and some have accounted for the observed catalytic effects, we do not yet have a consensus about the relative importance of different factors. One of the most intriguing proposals is associated with the idea that dynamical

Grant sponsor: NIH; Grant numbers: GM24492, 5U19A105010; Grant sponsor: NSF; Grant number: MCB-0342276; Grant sponsor: NCI; Grant number: 1 U19 1050101-01.
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Received 25 August 2009; Revised 5 October 2009; Accepted 16 October 2009
Published online 6 November 2009 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/prot.22654

effects contribute to enzyme catalysis. Although the definition of "dynamical effects" will require special discussion (see below), in a broader sense, it means that the enzyme has evolved to optimize a particular vibrational mode for moving the system to the TS, or for converting a system at the TS to the product state.

The general idea that dynamical effects play a major role in enzyme catalysis dates back at least 25 years.[2–5] It has gained momentum since the mid-nineties and continues to attract considerable attention.[6–19] Although this idea has been challenged,[20–26] it clearly has major appeal as is seen from its frequent appearance in high impact journals (see for instance Refs. 27–31 amongst others), as well as its repeated implication in countless recent papers (e.g., Refs. 15, 32–34). The situation in the field is still in a state of flux, where, in some cases, the dynamical proposal is stated more subtly (see below for examples). However, in general, many of the papers that have promoted the dynamical proposal have been rather vague about what is meant by this proposal, and, have sometimes avoided discussing works that have addressed the definition in a clear way. In fact a symptomatic example of this problem is a recent very high profile review[27] that described the most careful yet study of the dynamic proposal as a cause of "confusion" in the field. Now, our aim here is not to set the record straight, but rather to insist that any discussion of the issue starts with a well defined proposal, so that one will be able to apply the scientific methodology to examine whether or not this proposal is valid. In this respect, it seems to us that it is imperative to move away from soft definitions of the dynamical proposals (where we have observed a recent tendency to describe differing views of the catalytic role of enzyme dynamics as simply *semantic* issues). Fortunately, the key issues concern the catalytic mechanisms that have actually been proposed, and not what names have been used to describe such proposals. As we believe that most fundamental points about this issue rise considerably above the level of semantics, we will focus here on the specific proposals that have been put forward, as well as other possible logical definitions, and try to examine the validity of the dynamical proposals in the cases where there is *no question* what is meant by each proposal. In other words, we will analyze well-defined proposals in order to be able to determine their validity. Now it may seem to some that our definitions here are too narrow. However, we would like to remind the reader that definitions are, *per definition*, narrow, and only by using specific definitions is it possible to argue a case understandably and clearly for any audience. Additionally, we remind the reader that what is important is what the key players in the field have *actually* stated, not what may be later *claimed* was stated, when it starts becoming clear that the proposal has difficulties (as was the case, for instance, in the following exchange on the dynamic origins of allosteric activation[35]). This issue is central to the present work, as the dynamical proposal (which now seems to be accepted by many) has been put forward by some workers in such a way that it is obfuscated with vague statements, which make it hard to address in a logical and well-defined manner. Thus, we find it *crucial* to draw the attention of the reader to the documented opinions of the key workers in the field, based on the *published* research. Some such examples are highlighted below. We should note that these examples are merely a few (out of countless more) that are presented here in order to illustrate our point, and to prevent the discussion in the present work from being labeled as "semantic hyperbole," which would prevent a serious analysis of the validity of the dynamical proposal. We also like to clarify that the examples below are not meant to express any judgment on the issue (at this stage), but rather to simply establish what has been proposed, and to focus the discussion on factual proposals in order to prevent the discussion from becoming circular.

"Hidden Alternative Structures of Proline Isomerase Essential for Catalysis", *Nature* 462 (2009), 669–673. *A long-standing challenge is to understand at the atomic level how protein dynamics contribute to enzyme catalysis. X-ray crystallography can provide snapshots of conformational substrates sampled during enzymatic reactions, while NMR relaxation methods reveal the rate of interconversion between substates and the corresponding relative populations.*

[. . .] These studies introduce crystallographic approaches to define functional minor protein conformations and, in combination with NMR analysis of the enzyme dynamics in solution, show how collective motions directly contribute to the catalytic power of an enzyme.

"Linkage Between Dynamics and Catalysis in a Thermophilic-Mesophilic Enzyme Pair", *Nat Struct Mol Biol* 11 (2004), 945–949. *A fundamental question is how enzymes can accelerate chemical reactions. Catalysis is not only defined by actual chemical steps, but also by enzyme structure and dynamics. To investigate the role of protein dynamics in enzymatic turnover, we measured residue-specific protein dynamics in hyperthermophilic and mesophilic homologs of adenylate kinase during catalysis. A dynamic process, the opening of the nucleotide-binding lids, was found to be rate-limiting for both enzymes as measured by NMR relaxation. Moreover, we found that the reduced catalytic activity of the hyperthermophilic enzyme at ambient temperatures is caused solely by a slower lid-opening rate. This comparative and quantitative study of activity, structure and dynamics revealed a close link between protein dynamics and catalytic turnover.*

"Intrinsic Dynamics of an Enzyme Underlies Catalysis," *Nature* 438 (2005), 117–121. *This correlation suggests that the protein motions necessary for catalysis are an intrinsic property of the enzyme and may even limit the overall turnover rate. Motion is localized not only to the*

active site but also to a wider dynamic network. Whereas coupled networks in proteins have been proposed previously, we experimentally measured the collective nature of motions with the use of mutant forms of CypA. We propose that the pre-existence of collective dynamics in enzymes before catalysis is a common feature of biocatalysts, and that proteins have evolved under synergistic pressure between structure and dynamics.

"Instrinsic Motions Along an Enzymatic Reaction Trajectory," *Nature* 450 (2007), 838–844. *The mechanisms by which enzymes achieve extraordinary rate acceleration and specificity have long been of key interest in biochemistry. It is generally recognized that substrate binding coupled to conformational changes of the substrate-enzyme complex aligns the reactive groups in an optimal environment for efficient chemistry. Although chemical mechanisms have been elucidated for many enzymes, the question of how enzymes achieve the catalytically competent state has only recently become approachable by experiment and computation. Here, we show crystallographic evidence for conformational substates along the trajectory toward the catalytically competent "closed" state in the ligand-free form of the enzyme adenylate kinase. Molecular dynamics simulations indicate that these partially closed conformations are sampled in nanoseconds, whereas nuclear magnetic resonance and single-molecule fluorescence resonance energy transfer reveal rare sampling of a fully closed conformation occurring on the microsecond-to-millisecond timescale. Thus, the larger-scale motions in substrate-free adenylate kinase are not random, but preferentially follow the pathways that create the configuration capable of proficient chemistry. Such preferred directionality, encoded in the fold, may contribute to catalysis in many enzymes.*

"Enzyme Dynamics During Catalysis," *Science* 295 (2002), 1520–1523. *Although classical enzymology together with structural biology have provided profound insights into the chemical mechanisms of many enzymes, enzyme dynamics and their relation to catalytic function remain poorly characterized. Because many enzymatic reactions occur on time scales of microseconds to milliseconds, it is anticipated that the conformational dynamics of the enzyme on these time scales might be linked to its catalytic action.*

"A Perspective on Enzyme Catalysis," *Science* 301 (2003), 1196–1202. *The exploration for links between protein structure, movement, and catalysis will be expanded by the advent of new methods. We anticipate additional examples of enzymes for which motion is important and other cases for which it is not. Particularly attractive are techniques that permit the observation of single molecules on the same millisecond-to-second time scale on which enzymatic reactions normally occur. Such kinetics provide data for conformational changes during enzymatic turnover that may be masked in ensemble-averaged studies. Ensemble studies featuring isotopic editing of specific regions of the protein coupled with temperature jump relaxation also show considerable promise in detecting motions of mobile loops and active-site residues important to catalysis. Similarly, the extensive isotopic labeling of the substrate coupled with kinetic isotope effect analysis can provide structural information for reaction coordinate motions from the vantage point of the substrate. Isotopic editing, of course, remains the basis of Raman, infrared, and NMR studies of protein dynamics.*

The reader should note that, as stated earlier, the examples presented here are only brought up in order to prevent the argument that none of the aforementioned opinions have been expressed. Furthermore, although one can use technicalities to argue that the dynamical proposal is sometimes not explicitly stated, we will clarify this issue in the section on dynamical coherence effects. That is, we will show that this is the *only* logical (and intended) implication from the above (and related) statements.

The main focus of this perspective will be to critically examine the current state of the dynamical proposal, and particularly the impact of recent experimental and theoretical studies, though we will also include points considered in our previous reviews. As stated earlier, we will try to use clear definitions to remove any potential confusion due to semantic issues. We will start by discussing the effects about which there exists a consensus view, before moving on to other proposals where the juries are still out.

DEFINING ENZYME CATALYSIS

Before even considering what is meant by "dynamics," it is absolutely essential to be clear about enzyme catalysis, since the issue here is the idea that dynamical effects contribute to enzyme catalysis. The definition starts by considering the generic enzymatic reaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_{cat}} ES^{\ddagger} \rightarrow EP \rightarrow E + P \qquad (1)$$

where E, S, and P are the enzyme, substrate and product, respectively, and ES, EP, and $ES^{\ddagger}$ are the enzyme-substrate complex, enzyme-product complex and transition state. Since the apparent k_{cat} may reflect more complex kinetics, it might not always correspond to the rate constant of the chemical rate (which is usually referred to as k_2), but here, in most cases, we consider, k_{cat} as being equal to k_2. At any rate, as was shown convincingly by Wolfenden and coworkers,[36] many enzymes appear to have evolved to optimize k_{cat}/K_M, where $K_M = (k_{-1} + k_{cat})/k_1$. This optimization can involve maximizing k_{cat}, minimizing K_M, or both.

To quantitatively evaluate enzyme catalysis, we first must ask "*catalysis relative to what?*" The most obvious reference is the uncatalyzed reaction in water. As the mechanism of the reaction can be different in water than in the enzyme, one must not only consider the effects of altering the environment, but also that of any changes in

the mechanism. However, differences in mechanism (such as using a general base instead of water as a base) can be classified as "chemical effects," and such effects are well understood. Thus, we find it very useful to consider the "chemically filtered" reference reaction.[37] We also frequently consider the reference reaction at a "solvent cage," where the fragments are free to move within a typical volume of about 3 Å^3 (this leaves only the trivial effect of moving from the cage to a molar volume to evaluate analytically, which simplifies the overall analysis[38]). Here, the key question is how to introduce the proper volume restraint in order to keep each of the fragments in the system within v_{cage}. This is achieved by applying a special cage restraint (K_{cage} = 0.3 mol^{-1} Å^{-2}) to a single atom in each fragment in the system, thus keeping the fragments within a restricted volume (see Ref. 38 for more details), and then analytically evaluating the free energy associated with releasing the cage restraint and allowing the fragment be in the full molar volume.[38,39] Note that the cage effect has been well understood in terms of the effect of moving to 55M[40] and was never a puzzling factor in catalysis. Our reference, therefore, should be a reaction that occurs by the same mechanism as in water, so that the key question becomes how the structured environment of the enzyme accelerates the reaction relative to the same process in a solvent cage.[41]

Any proposal of dynamical contributions to catalysis *must* focus on the rate constants k_{cat} and k_{cat}/K_M. Thus, we first have to clarify the nature of the rate constants. Our starting point is the well-known expression:

$$k = \kappa k_{TST} \tag{2}$$

where k_{TST} is the rate constant from transition-state theory (TST):

$$k_{TST} = \frac{1}{2}\langle|\dot{x}|\rangle_{TS}\exp[-\Delta g^{\ddagger}\beta]\Big/\int_{-\infty}^{\ddagger}\exp[-\Delta g(x)\beta]dx \tag{3}$$

and κ is the "transmission coefficient." In Eq. (3), $\beta = 1/k_BT$, where k_B is the Boltzmann constant and T is the absolute temperature, x represents a generalized reaction coordinate, which we now consider to be a function of time; $\dot{x}$ is the time-dependent velocity along x, $x^{\ddagger}$ is the (time-independent) value of x at the TS, $\langle\cdots\rangle_{TS}$ denotes a time average over periods in the region of the TS, and $\Delta g^{\ddagger}$ is the activation free energy, $\Delta g(x^{\ddagger})$.

In TST, the average velocity in the TS, $\langle|\dot{x}|\rangle_{TS}$, is equated to the mean velocity for one-dimensional translation in a thermally equilibrated system, by the relationship shown in Eq. (4):

$$\langle|\dot{x}|\rangle_{TS} = (2\beta^{-1}/\pi m)^{1/2} \tag{4}$$

where m is the reduced mass for this motion. Finally, if one includes the additional assumptions that Δg is a harmonic function of x and that translation along x is an equipartition of the energy with the other motions of the system, k_{TST} can be further simplified to give:

$$k_{TST} \approx (\beta h)^{-1}\exp(-\beta\Delta g^{\ddagger}) \tag{5}$$

In light of the discussion earlier, it is clear that chemical catalysis would mean that the rate constant of Eq. (2) is different to the rate constant of the reference reaction in solution. We can also ask why k_{cat}/K_M is different to the rate constant for the catalyzed reaction, but this will include the rather trivial issue of the binding step, which has *never* been a puzzling part of the problem of enzyme catalysis. Thus, we will focus on the origin of the difference between k_{cat} (or k_2) and k_{cage}. Furthermore, we will focus on the chemically filtered reference reaction discussed earlier, though we should emphasize, however, that we have no problem to convert the obtained result to the actual rate in solution. Regardless of the definition used, the question we have to address is whether dynamical effects in any way aid in making k_{cat} faster than k_{cage}.

DEFINING DYNAMICAL EFFECTS

As clarified earlier, we are looking for dynamical contributions to catalysis. However, this question is meaningless without defining what dynamical effects actually *are*. In this work, we cover a large range of possible reasonable definitions that have been used in the literature, in order to avoid leaving the impression that our conclusions depend on the definition used. As atoms move in any chemical process that occurs at above a few °K, we cannot equate dynamics with the trivial fact that atoms are moving. More specifically, if the rate constant is entirely determined by the probability that the system will reach the TS, then we do not have dynamical effects. In such a case, the odds that the atoms are at different positions is determined by the corresponding Boltzamnn probability, and, in such a case, TST is fully valid.

Thus, dynamical effects require deviations from TST. In principle, it is possible to show that all the dynamical effects can be grouped into the transmission factor,[42–44] and having dynamical effects requires that the transmission factor is significantly smaller than one (note that we will nevertheless consider other definitions as well in this work). Another very reasonable (and perhaps the best) definition of dynamical effects is that the probability of reaching the TS does not follow the Boltzmann factor. In this way, we have coherent non-Boltzmann motions that can be classified as genuine dynamical effects.

Some workers may try to classify deviations from TST as being due to nuclear tunneling. We would like to clarify that tunneling corrections to TST are well

understood, and are frequently considered as to be a reduction of the activation free energy.[45] Nevertheless, we will consider this proposal in the present work. Other workers imply that having coupled motions is associated with dynamical effects. Here, it is important to clarify that there is nothing special about having coupled motions, since almost *all* motions in condensed phases are coupled, though it seems that overlooking this fact has led some to assume that coupled motion represents a dynamical effect. At any rate, having a coupled motion, along a reaction coordinate that follows the Boltzmann probability is *not* indicative of any dynamical effect. Furthermore, we must consider the fact that the motions in solution are *also* strongly coupled. Thus, to invoke coupling contributions to catalysis, one has to show that the coupling in the enzyme is fundamentally different from that in solution, as only then is it relevant to the rate enhancement by the enzyme.

In summary, one can invoke dynamical effects when (a) the transmission factor is small, (b) there exist non-Boltzmann coherent motions, (c) there are large deviations from TST and (d) the mode of the coupling in the enzyme does not follow the Boltzmann distribution. However, the problem with all of these proposals is that even if one does find some dynamical effects, they have to be very different in solution and in the protein in order to be considered relevant to catalysis. At any rate, once we have clearly defined the dynamical proposals, we can then examine their validity.

SIMULATING THE RATE CONSTANTS OF ENZYMATIC REACTIONS

Although experimental studies provide crucial information about the magnitude of the catalytic effect, it is hard to determine whether the given rate acceleration reflect some dynamical effects (or, in fact, any other effect) by experiment alone. Perhaps the only way to determine what the magnitude of a given dynamical effect actually *is*, is to calculate this effect (though naturally doing so by a model that reproduces the observed rate enhancement). However, in order not to distract the reader from the main issue, we have left the technical discussion of this issue for the section "Technical Background" and for the literature considered in this section.

DYNAMICAL EFFECTS IN ULTRAFAST REACTIONS

Before addressing the idea that dynamical effect contribute to enzyme catalysis, it is useful to consider some specific studies of dynamical effects.

Ultrafast photoisomerization and electron transport reactions

Studying frictional effects in excited state ultrafast reactions has been a field of general interest, for instance in the case of stilbene,[46,47] where it was found that frictional effects are generally small when the barrier is large. A related study is one of the femotosecond photoisomerization of bacteriorhodopsin (bR),[48] which was pumped at either 550 or 405 nm and probed between 640 and 950 nm. This study found the shape of the simulated emission spectra to be time independent (which is inconsistent with more conventional models that invoke low-frequency motion out of the Franck-Condon region following excitation). This result is similar to the photoisomerization of *cis*-stilbene, and demonstrates that there exists potentially interesting dynamics in these ultrafast reactions.

Simulations of ultrafast photoisomerization have a long history, dating as far back as the simulations of the primary step in the vision process[49] in the 1970s. Significant progress has been made on this front since then (e.g., Refs. 50–54) and, particularly, recent theoretical and experimental studies strongly suggest that subpicosecond isomerization[50,54,55] can have significant dynamical effects.

Another clear case of dynamical effects is provided by the primary electron-transfer step in photosynthetic bacterial reaction centers. In the photosynthetic case, an ensemble of molecules can be coherently excited by use of a short pulse of light. In this system, electron transfer from the excited state occurs on the same timescale as relaxation among the solvent modes that are coupled to the reaction. Vibrational coherence can result in oscillatory kinetics, and deviations from the predictions of Marcus theory.[56–59] Related dynamical effects have also been observed in ground-state organic reactions that proceed from an instantaneously generated intermediate.[60]

Electrostatic fluctuations and solvent relaxation times

Theoretical studies of solvation dynamics in enzymes have been a major part of the analysis of dynamical ideas.[25,61,62] Such studies have included our early determination of the autocorrelation of the electrostatic energy gap in enzymes and solutions (e.g., Ref. 26), and an analysis of the corresponding relaxation time (similar simulations have been recently conducted with Hynes's frictional approach,[62] which is equivalent to the energy gap approach). This energy gap can be separated to the contributions from the reacting system (solute) and the surrounding (solvent). The solvent contribution is of special interest, since this is where the reaction in the enzyme and in solution can be different. This contribution is also strongly related to the key parameters in experimental studies of solvation dynamics. For example, Fig. 1 shows the calculated fluctuations of the energy gap

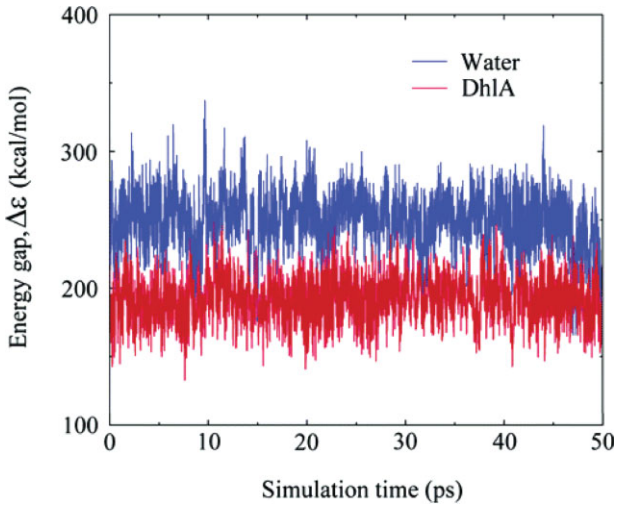

Figure 1

The energy gap between the diabatic product and reactant states in the reaction catalyzed by haloalkane dehalogenase (DhlA), during MD simulations of the enzyme (red), as well as the same reaction in water (blue). This figure was originally presented in Ref. 63.

between the reactant and product states during MD simulations of haloalkane dehalogenase and the reference system in water.[63]

As can be seen from Fig. 1, the fluctuations of the solvent coordinates in the enzyme and solution are quite similar. For a more definitive analysis, one can use the autocorrelation function of the energy gap to compare the transmission coefficients of the enzymatic and solution reactions. Fig. 2 shows the autocorrelation functions of the energy gap in the region of the TS for haloalkane dehalogenase as well as for the reference reaction. This figure presents the autocorrelation functions of both the total energy gap, $C(t)$, as well as the electrostatic component, which we take as the solvent coordinate, $C_{el}(t)$. It includes the results from two MD simulations of each system, in order to show the variability of the results. Although the results depend somewhat on the initial positions and velocities in the trajectories, the decay kinetics of the autocorrelation function are very similar in the enzyme and water, indicating that the transmission coefficients are not significantly different in the two systems. In both cases, the system relaxes in about 1 ps. Direct simulations of the actual relaxation from the TS to the product state[25,64] give similar results to those obtained from the energy gap, and these simulations give no indication that the enzymatic catalysis depends strongly on dynamical effects.

At this point, it may be useful for the reader to expand the discussion about the dehalogenase test case, which involves the nucleophilic attack of a carboxylate group on the carbon of chloroethane, *via* an S_N2 mechanism. Here, it has been demonstrated that the fluctuating dipoles of the solvent or protein are capable of either stabilizing or destabilizing the product state relative to the reactant state, thus modulating the chance that the solute moves to the product state[24] (a point that has been illustrated for many other systems[20] as well). The same system has also been examined by Nam *et al.*,[19] who used a QM/MM molecular orbital approach that focuses on the force autocorrelation approach [i.e., $C_F(t)$], which is a valid but less direct measure of the solvation dynamics of the autocorrelation function of the energy gap [$C(t)$].

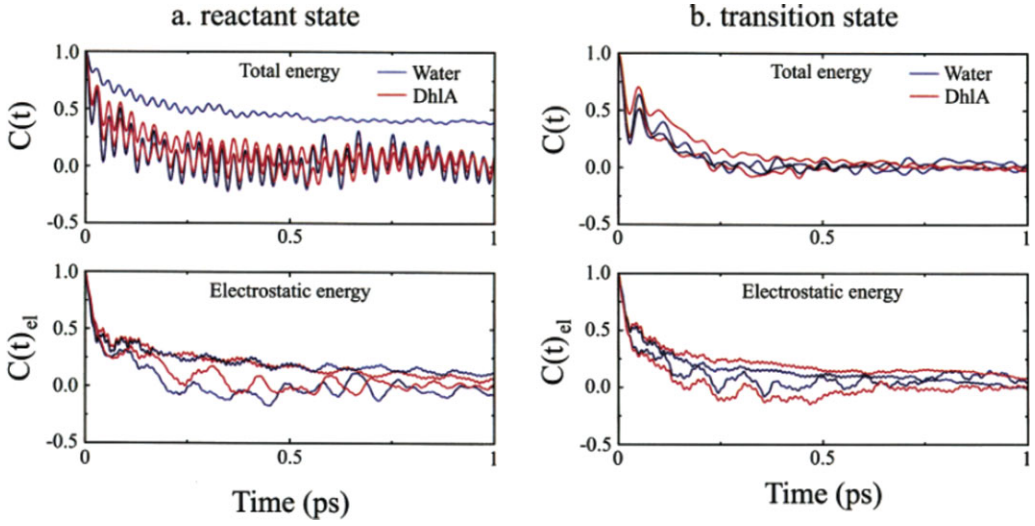

Figure 2

Autocorrelation function of the energy gap between the reactant and product states in the region of the TS in haloalkane dehalogenase (red), as well as the reference reaction in water (blue). The plot on the top shows the total energy, whereas that on the bottom shows only the electrostatic contribution to the energy. The autocorrelation functions are normalized to 1 at 0 time. This figure was originally presented in Ref. 63.

Here, Nam *et al.* found that $C_F(t)$ decays more rapidly in the enzyme than in water, and that in the enzyme, $C_F(t)$ has some oscillatory components that were not seen in water. The finding that $C_F(t)$ can be somewhat different in the enzyme and in water was already described in an earlier study of alcohol dehydrogenase,[20] although the solvation dynamics in the two systems was found to be similar. Additionally, Ref. 19 does not provide a separate analysis for the solute and solvent coordinates, which is difficult to do in standard QM/MM studies. However, the solute contribution cannot be reliably obtained by simply omitting the solvent's electrostatic contribution from the QM/MM Hamiltonian, as this reproduces the gas-phase results, which are generally quite different from the behavior of the solute in solution (this issue has been discussed at length in Refs. 61 and 64. In comparison, we have examined not only the haloalkane DhlA reaction, but also the reference system in water[63] (see Figs. 1 and 2), and have used the autocorrelation of the energy gap in order to compare the transmission coefficients in both the enzymatic and in the solution reactions. Our studies demonstrated that even though the precise results obtained depend somewhat on the initial positions and velocities in the trajectories, the decay kinetics of the autocorrelation functions are very similar both in the enzyme and in water, indicating that the two systems have very similar transmission coefficients. It should be pointed out that it has also been possible to examine this system by direct simulations of the actual relaxation from the TS to the product state.[25,64] However, overall, none of these simulations have given any indication that enzymatic catalysis is dependent on dynamical effects.

The possible relevance of solvation dynamics to enzyme catalysis involved experimental examination of the solvation dynamics at the active site of an enzyme, glutaminyl-tRNA synthetase (GlnRS), was studied using a fluorescence probe, acrylodan, site-specifically attached at cysteine residue C229 near the active site.[65] The picosecond time-dependent fluorescence Stokes shift indicates that in the absence of any substrate, there is slow solvation dynamics at the active site of the enzyme. This serves as a strong argument against the idea that the dynamics of the enzyme is faster than that of the solvent in the corresponding reference reaction.[66] Here, we see that the dynamics in the enzyme is, if anything, slower rather than faster than the corresponding solvent dynamics, a point which had already been put forward by earlier theoretical studies (e.g., Ref. 63).

DO DYNAMICAL COHERENCE EFFECTS PLAY A ROLE IN ENZYME CATALYSIS?

While some may argue about the technicalities of what precisely constitutes dynamical contributions to catalysis, it is clear that when the rate constant involves coherent fluctuations that do not follow the Boltzmann law, we *do* have dynamical effects. Thus, it is important to examine whether enzymatic reactions may involve coherent fluctuations. As pointed out in the previous section, coherent dynamical effects do occur in photobiological processes such as the primary event in the vision process, and in the primary electron-transfer step in photosynthetic bacterial reaction centers. However, such effects are far less likely to operate in thermally activated barrier-crossing events, where the equilibrium energy distribution usually appears to determine the rate constant.

In order to be very clear about the implications of the dynamical proposal, we clarify it by means of Fig. 3. At this point we would like to insist that this is what is *actually* being implied by the dynamical community (see also the quotes from key papers presented in the "Introduction" of this review). In this respect, it is important to recognize that the dynamical proposal is very rarely presented with a clear definition of what is actually meant, which perhaps allows some to later argue that no long-range memory effects or non-Boltzmann behavior were ever suggested. The aim of this review is to prevent the confusion that can be caused by such arguments, and to clarify the fact that the dynamical proposal has clear and unique implications *regardless* of whether these implications have been clearly stated or not. For example, let us consider statements like "*During catalytic action of the enzyme cyclophilin A, we detect conformational fluctuations of the active site that occur on a time scale of hundreds of microseconds. The rates of conformational dynamics of the enzyme strongly correlate with the microscopic rates of substrate turnover*"[15] or "*Molecular dynamics simulations indicate that these partially closed conformations are sampled in nanoseconds, whereas nuclear magnetic resonance and single-molecule fluorescence resonance energy transfer reveal rare sampling of a fully closed conformation occurring on the microsecond to millisecond timescale. Thus, the larger-scale motions in substrate-free adenylate kinase are not random, but preferentially follow the pathways that create the configuration capable of proficient chemistry. Such preferred directionality, encoded in the fold, may contribute to catalysis in many enzymes.*"[29] (amongst other works that claim that such slow motions contribute to catalysis, such as those of Refs. 28, 34, 70). Such statements cannot be formulated scientifically unless they involve non-Boltzmann behavior. Similarly, any suggestions that dynamics modulates the free energy surface (as in e.g., Ref. 71) have no clear meaning - motions do not tune any surface: any motion is a result of the surface itself, unless of course the dynamical idea were to be correct (which, as we discuss in this review, we doubt is the case). Thus, we like to insist on using the description of Fig. 3 as the main definition of the meaning of the dynamical proposal, and would gladly wait for a logical definition that formulates this proposal without the features of this figure.

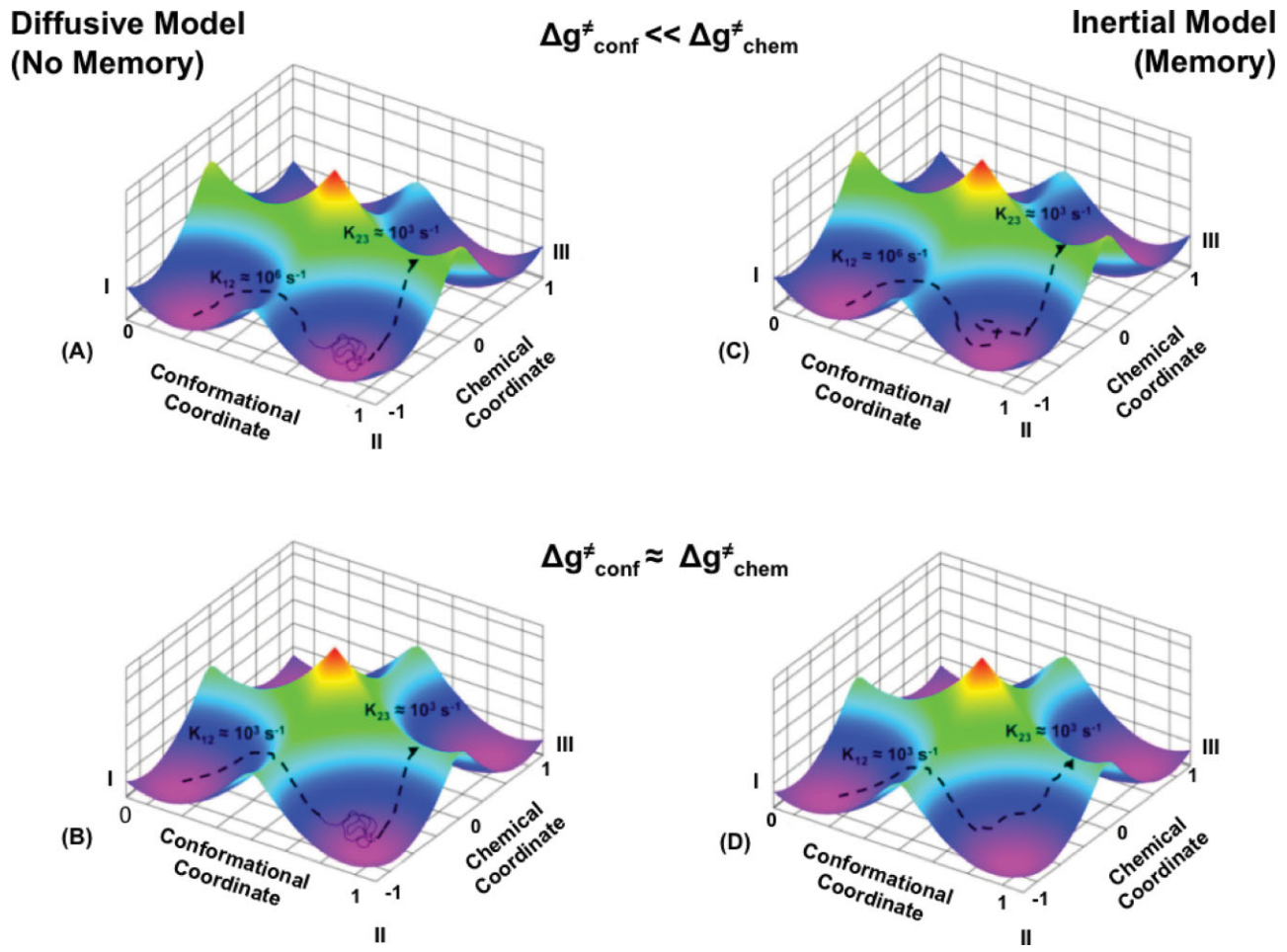

Figure 3

A schematic depiction of the diffusive (**A**, **B**) and the inertial models (**C**, **D**). These two limiting models are shown in the case where the conformational barrier is much smaller than the chemical one (i.e., $\Delta g^{\neq}_{conf} \ll \Delta g^{\neq}_{chem}$, parts A and C at the top of the figure), and where the two barriers are similar (i.e., $\Delta g^{\neq}_{conf} \approx \Delta g^{\neq}_{chem}$, parts B and D at the bottom of the figure). This figure was originally presented in the supporting information of Ref. 67.

Now, short timescale dynamical effects can be studied theoretically in a *direct* way by monitoring productive trajectories on the solute-solvent coordinate system. This can be achieved by propagating trajectories both forwards and backwards in time from the TS. Such a procedure was, for instance, carried out in the case of the haloalkane dehalogenase reaction.[63] This work demonstrated that the dynamics in both the enzyme and solution are incoherent, with the trajectories moving randomly in the reactant state, and occasionally acquiring enough thermal energy to move to the TS (see Fig. 4). Also, the overall displacement on the solvent coordinate is larger in water than in the enzyme. This is a reflection of the preorganization effect, and not a dynamical effect. Furthermore, the dynamics of the relaxation from the TS to the product state are essentially the same in the enzyme and solution.

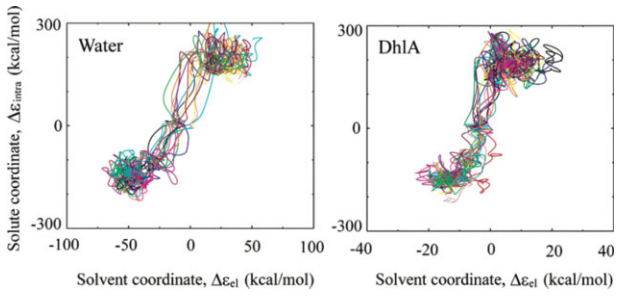

Figure 4

The behavior of 200 fs downhill trajectories run on the ground-state EVB surface of (**A**) the relevant reference reaction in water and (**B**) the DhlA system. This figure shows the trajectories separated into solvent and intramolecular solute components, and the time reversal of these trajectories corresponds to the actual reactive trajectories. This figure was originally presented in Ref. 63.

Unfortunately, most of the recent implications about dynamical effects are related to much longer timescales (see for instance the statements in the introduction of this review). Thus, we have a major challenge for both theoretical and experimental studies. Experimentally, it is necessary to show whether the slow conformational dynamics have any effect on the rate constant of the chemical step, while theoretically it is essential to run simulations of the system in up to the millisecond timescale, and this has been extremely challenging for current simulation approaches. The sections below will examine what has been learnt about this issue.

The experimental search for the dynamical coupling between the conformational and chemical coordinates

In recent years, nuclear magnetic resonance spectroscopy has been utilized to study the motions of enzymes over a wide range of timescales.[15,72–74] An interesting such case is that of the enzyme cyclophilin A (CypA), which catalyzes the *cis-trans* isomerization of peptidyl proline bonds.[15,74–76] Interestingly, the transverse relaxation of Arg55 (which is hydrogen bonded to the substrate and is essential for catalysis) has been demonstrated to accelerate in the presence of the substrate at a rate that approximately matches the dynamics for forming the TS, suggesting that the motions of this residue might play a dynamical role in the catalytic mechanism.[15] When considering this proposal, it is important to bear in mind that if Arg55 (or any other residue) moves along the reaction coordinate, and if its position changes in the TS, it necessarily moves on the same timescale as in the overall reaction. For example, the solvent molecules in a reaction in solution *must* rearrange during the reaction, and so must move at more or less the same rate as the solute atoms. Most of the reorganization of the environment will occur on the same timescale as that of the reaction, and the motions of the protein residues near the reacting substrate are not fundamentally different in this regard to the motions of the solvent molecules in solution. Furthermore, as long as the motions of the protein residues follow Boltzmann's law, they simply reflect probabilistic effects, and not *bona fide* dynamical effects (for a related misunderstanding, see also Ref. 72).

The dynamics of the reaction of CypA was also examined in both its substrate-free state and during catalysis,[15,77,78] and it was argued for a dynamic network of coupled motions along the protein, even in the absence of the substrate, that they claimed is essential for catalysis. More specifically, it has been suggested[15] that "*the characteristic enzyme motions during catalysis are already present in the free enzyme with frequencies corresponding to the catalytic turnover rate*" and that "*this correlation suggests that the protein motions necessary for catalysis are an intrinsic property of the enzyme.*" The aforementioned

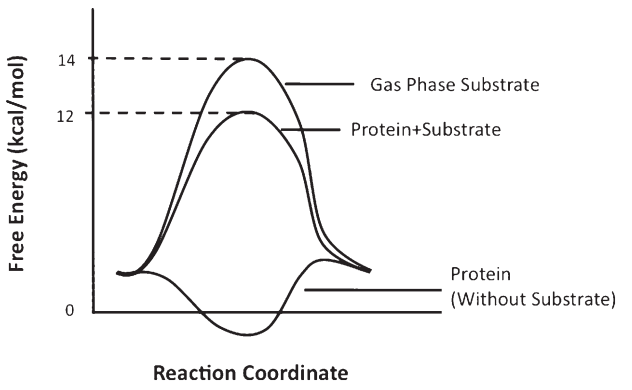

Figure 5

Showing why the trend in the energy contributions along the reaction coordinate of the reaction of CypA, contradict the proposal of Refs. 15 and 77. As seen from the figure, the chemical barrier in the protein (which is around 15 kcal/mol) is smaller than that in vacuum (see Ref. 75. Note also that these free energy values are very qualitative, and only brought in, in order to focus the discussion). This means that the contributions of the protein at the TS of the protein/substrate complex, is negative. Thus, if the barrier due to the protein along the chemical coordinate is around 15 kcal/mol in the apo-enzyme (as is implied by Kern's work), it cannot be similar to the same contribution at the ES system. This means that the study of the apo-enzyme is either (a) unlikely to tell us about the protein contribution along the chemical coordinate in the apo-enzyme, or (b) that the landscape in the corresponding barrier in the apo-enzyme is not related to the barrier in the presence of the substrate.

statement has very problematic implications. That is, if motion along the relevant chemical reaction coordinate in the absence of the substrate is as slow as the turnover time, it would mean that we already have a large barrier along the direction of the chemical reaction coordinate even in the absence of the chemical barrier and thus the sum of chemical barrier and the protein reorganization barrier must be larger than the actual turnover barrier, which is of course not the case. In other words, this time cannot be the same as the chemical reaction time since the barrier along the reaction coordinate is not the same in the absence and the presence of the substrate (note that the aforementioned statement implies that the protein contribution is the same in both cases). Obviously, it is hard to understand why motions between two states that are involved in a reaction should contribute to catalysis if such motions occur already relatively slowly in the absence of the substrate (the low speed implies a preexisting barrier for the reaction). A *truly* catalytic apoenzyme should, in the absence of the substrate, have free energy minima corresponding to the reactant and product states that are close together along the reaction coordinate in order to minimize the reorganization energy (see Fig. 5). Already having slow motions (on a scale that is close to the chemical reaction time) along the reaction coordinate in the absence of the substrate means that the reorganization energy was *not* minimized, and thus that

the apo-enzyme has not optimized the reaction speed (which is the opposite of the catalytic implications). Of course, the resolution is simple, and what is probably similar with and without the substrate is the barrier to the conformational change, which has very little to do with the chemical barrier. It is likely that either (a) the NMR measurement with the apo-enzymes does not provide the relevant information about the rate of movement in the direction that will become the chemical reaction coordinate, but rather only along the conformational coordinate, or (b) the protein contribution along the chemical barrier in the apo-enzyme has nothing to do with the corresponding barrier at the ES complex.

Benkovic, Wright and coworkers[9,79,80] have studied the reaction of dihydrofolate reductase by NMR. They found that site-directed mutations of 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 coworkers,[12,81] who carried out MD simulations of three ternary complexes of the enzyme. Motions of some residues were strongly correlated, and were different in the enzyme-substrate and enzyme-product complexes. Some of these motions were modified in simulations of mutant enzymes with diminished activity. However, these studies did not examine any of the transition states in the reaction or demonstrate any dynamical effects on the rate constant. The different motions of the ES and EP complexes could just reflect the coupling of enzyme-substrate interactions to interactions of various groups in the protein, which is common to all enzymes.

NMR studies have also been used in order to attempt to examine the dynamic energy landscape of enzymes such as dihydrofolate reductase. For instance, Wright *et al.* used NMR[71] in order to characterize higher energy conformational substrates of *E. coli* dihydrofolate reductase. Each intermediate in the catalytic cycle was found to sample low-lying excited states with conformations that resemble the ground-state structures of preceding and subsequent intermediates, with substrate and cofactor exchange occurring through these excited substrates, suggesting that the maximum hydride transfer and steady-state turnover rates are governed by the dynamics of the transitions between the ground and excited states of the intermediates. Thus, the authors have argued that the bound ligands modulate the free energy landscape in order to funnel the enzyme through its reaction cycle along a preferred kinetic path. As stated earlier, the modulation of the landscape is *not* a dynamical effect, and claiming that ligand binding changes the landscape (*via* an induced fit model) does not provide any insight into catalysis, since the important question is how the barrier in the ES complex (whose formation might well reflect a different structure than without the substrate) is reduced relative to that of the reacting complex in water. The implications of the arguments about funnel effects with respect to the landscape will be analyzed in more detail in section "Free Energy Landscapes and Catalysis."

Finally, to complete this section, we would like to focus on the adenylate kinase system, as this enzyme has been the subject of extensive experimental studies of the dynamical proposal.[29,82] For instance, several workers have recently simulated or observed thermally driven dynamics on different timescales, and on this basis have argued for a link between μs to ms domain motions and enzymatic function in various systems[34,68–70,83] (despite the fact that not much is understood about the connection between such motions and local atomic fluctuations, which tend to be much faster). As an example of this, Kern and coworkers[28] have studied fluctuations in the hinge regions of adenylate kinase and have demonstrated that while ps to ns timescale atomic fluctuations in this region are quite different between a mesophilic and a hyperthermophilic adenylate kinase, they are very similar at temperatures where the enzymatic activity and folding energy are matched. Thus, based on this, the authors have argued for a connection between the different timescales and the corresponding amplitudes of motions in adeylate kinase in a so-called "hierarchy of timescales," and believe that this hierarchy and its linkage to catalytic function is likely to be a general characteristic of protein function.

It should be noted that while these studies are extremely valuable, they have not actually established whether or not the conformational motions can transfer energy to the chemical coordinate. More specifically, despite major experimental effort and the publication of multiple articles in high profile journals, there is no single study that actually establishes any connection between the conformational dynamics and the change in the rate of the chemical step. At most, we have studies that have found a similar time range for the chemical and conformational steps, but this does not provide any proof that the two are correlated. Thus, we must explore this issue by theoretical approaches and such studies will be considered in detail in section "Consistent Theoretical Studies Have Established That There Is No Dynamical Coupling Between the Chemical and Conformational Coordinates."

Single molecule experiments

In recent years, advances in room-temperature single molecule spectroscopy have allowed for the dynamic behavior of individual molecules to be recorded in real time.[84–88] In turn, this has resulted in an increase in interest in the relationship between single-molecule experiments and the nature of protein landscapes. For instance, Xie and coworkers[89] have examined the enzymatic turnovers of single cholesterol oxidase molecules in real time by monitoring emission from the enzyme's fluorescent

active site, flavin adenine dinucleotide (FAD), and noticed significant and slow fluctuation in the rate of cholesterol oxidation by FAD. They then used single-molecule approaches to examine the static and dynamic disorder of the reaction rates, and used this to argue for a molecular memory phenomenon in which an enzymatic turnover was not independent of its previous turnovers due to a slow fluctuation of protein conformations. Thus, this[89] and subsequent single-molecule studies (e.g., Refs. 90–92) have proposed that a single enzyme molecule will exhibit large temporal fluctuates of the turnover rate constant over a wide (1 ms–100 s) range of timescales. This phenomenon has been broadly referred to as "dynamic disorder."[93,94] It has been argued that the existence of such slow conformational changes on the same timescale of enzymatic reactions makes the application of transition state theory inadequate[95] (even though transition state theory has actually been demonstrated to provide an excellent tool for studies of enzymatic catalysis[96]).

Xie and coworkers[97] have also used fluorescence assays in order to examine conformational changes within bacteriophage T7 DNA polymerase ternary complex upon the binding of a dNTP substrate. The authors found that the binding-induced conformational change is much slower in the case of the wrong base pair (W) than for the right (R) base pair, and they therefore suggested that the conformational change plays an important role in controlling the fidelity. They also observed that the bimolecular association rate constant (k_{on}) is correlated with the binding energy. The finding in this article is very important and adds crucial information to the overall picture of DNA fidelity. However, we find some aspects of the authors' interpretation of their results slightly problematic. That is, they suggest that the overall barrier that corresponds to k_{cat}/K_M determines the fidelity, and if the barrier that corresponds to the rate of the binding step (k_{on}) is smaller than the overall barrier, then k_{on} cannot contribute to the fidelity. In fact, the important finding that k_{on} is correlated to the binding energy leads to another interesting option, i.e., that the fidelity is most probably controlled by the difference between the overall barriers for R and W. This barrier is determined by two contributions—the binding free energy (rather than the barrier in the binding step) and the activation barrier for the chemical step (see Ref. 98). To achieve high fidelity, it is important for the enzyme to decrease the binding free energy (i.e., make it less negative) as well as to increase the chemical barrier upon going from R to W. The decrease in the binding energy leads to an increase in the binding barrier, as a result of the correlation discovered in Ref. 97. The increase in K_M for *W* is part of the effect that is needed to achieve high fidelity. Thus, the increase in the binding barrier is a result of the factors that control fidelity, rather than the reason for fidelity.

Based on the single molecule observations, there have been attempts to test the Michaelis-Menten equation at the single-molecule level,[95,99] and a recent work constructed a two-dimensional multisurface reaction free energy description of the catalytic cycle that explicitly connects multitimescale conformational dynamics and dispersed enzymatic kinetics to the classical Michaelis-Menten equation[95] (though this work did not make any novel contribution). Here, the models presented the surface function of a slow conformational motion on a collective enzyme coordinate, Q, that facilitates the catalytic reaction along the intrinsic reaction coordinate, *X*, thus providing a dynamic realization of transition-state stabilization. The catalytic cycle was subsequently modeled as transitions between multiple displaced harmonic wells in the *XQ* space (representing different states of the cycle), which was then constructed according to the free energy driving force of the cycle. Based on such a setup, the authors proposed that the enzyme-substrate complex under strain will exhibit a nonequilibrium relaxation toward a new conformation that lowers the activation energy of the reaction, thus enslaving the chemical reaction in *X* to the down hill slow motion on the *Q* surface. Unfortunately the hypothetical surface was not based on any actual enzyme landscape and had an insignificant barrier along the chemical coordinate. In fact, as was pointed out in Ref. 100 there is no evolutionary pressure to reduce the chemical barrier for an enzyme-catalyzed reaction much below the diffusion controlled limit. As a result, it is unlikely that the chemical barrier will ever fall significantly below the binding barrier, and thus even a hypothetical situation in which the chemical barrier is much smaller than the binding barrier[95] is highly unrealistic (see section "Consistent Theoretical Studies Have Established That There Is No Dynamical Coupling Between the Chemical and Conformational Coordinates").

Consistent theoretical studies have established that there is no dynamical coupling between the chemical and conformational coordinates in cases with a significant chemical barrier

As was clarified in the discussion above, there is no current experimental finding that shows a clear relationship between the conformational dynamics and the dynamics of the motion along the chemical coordinate. In the absence of experimental evidence, the only way to actually examine the dynamical hypothesis is to use theoretical studies that simulate the long timescale dynamics along the conformational and chemical coordinates. Such studies are extremely challenging however, and we have only recently started to make progress on this front.

We took a major step in this direction in a recent simulation study[67] that could actually explore the dynamical idea, by bridging the necessary timescale for examining

the dynamical coupling between the conformational and chemical motions. This was achieved by means of a multiscale approach, which allowed for the exploration of the dynamical nature of enzyme catalysis in the ms timescale, making it the first realistic simulation-based analysis of the proposed dynamical coupling on such a long timescale. Our approach is based on renormalizing lower-dimensionality models in such a way that they capture the energetics and dynamics of the full reacting protein system by transforming the energetics and dynamics of the explicit all-atom enzyme to an equivalent low-dimensional system. Some elements of this approach have been introduced in our early works (e.g., in Ref. 101), but this is the first work that uses this approach for simulating the long timescale behavior of enzymatic reactions. The full technical details of this approach are presented in Ref. 67 and here we will only be discussing the major findings of the study of Ref. 67.

In Ref. 67, we used the catalytic reaction of adenylate kinase as a benchmark, though we also demonstrated that our approach can be applied to a general protein model with different types of conformational motions. Our preliminary study showed that the kinetic energy of the conformational motion is completely dissipated during the opening and closing of the active site lid of adenylate kinase, and thus that this *cannot* affect the time of the chemical process (a situation that holds for as long as the chemical barrier is higher than a few kcal/mol). That is, we found that there is no significant dynamical coupling between the chemical and conformational trajectories, since the inertial part of the conformational motion decaying very rapidly (i.e., on a timescale of far less than a nanosecond), and the remainder of the process being completely guided by the free energy surface and the corresponding Boltzmann probability. Finally, the chemical process was shown to have no significant "memory" of the dynamics of the conformational motion. This crucial finding is illustrated in Fig. 6. We must also clarify that Ref. 67 not only explored the dynamical proposal with the renormalized 2D model, but also with the full CG protein-substrate model, where it was demonstrated that adding very large excess kinetic energy to the conformational motion (resembling the

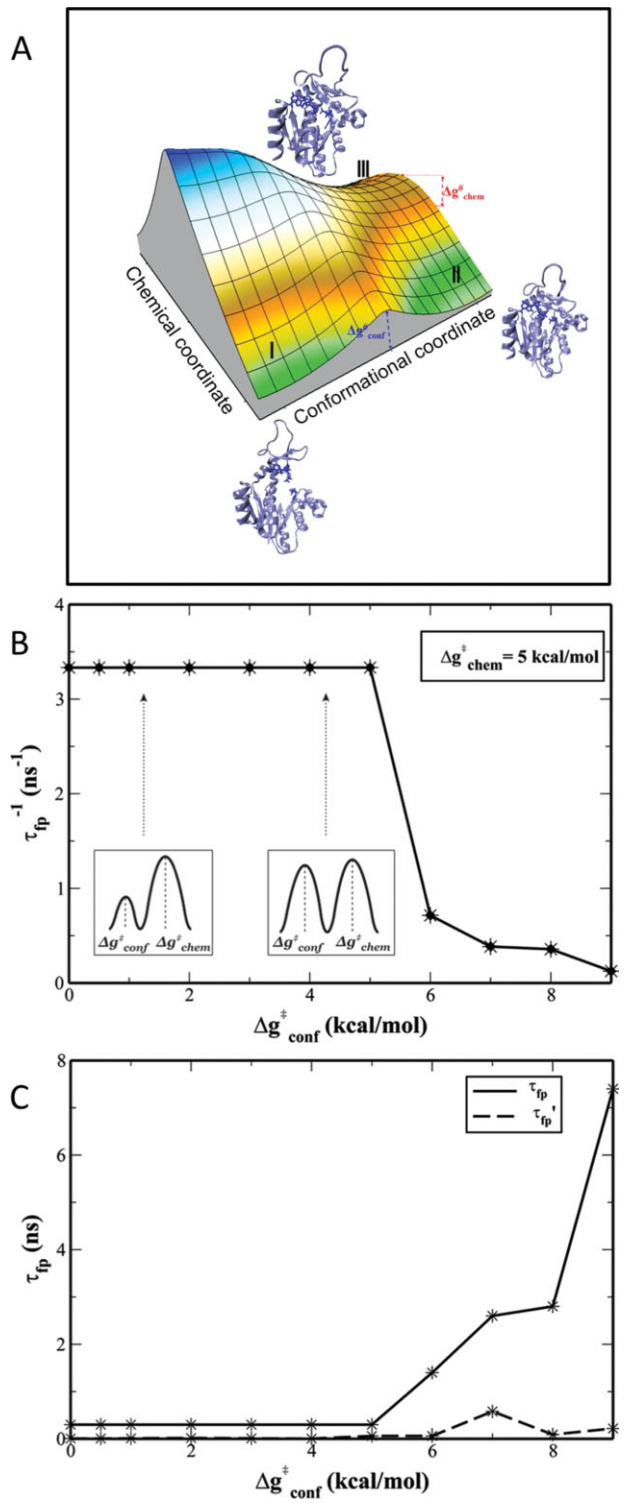

Figure 6

Examining the coupling time between the conformational and the chemical dynamics, showing (**A**) the actual effective 2D free energy surface for the ADK system and the meaning of the two coordinate set, and (**B**) and (**C**) the relationship between the first passage (fp) time, τ_{fp}, over the chemical barrier, and the height of the conformational barrier (here we consider a hypothetical case where $\Delta g^{\neq}_{chem} = 5$ kcal/mol, but the same results are even more likely to hold for the real larger barrier). The calculations represent the average from several runs. Here, two correlations are considered: (**B**) The inverse fp time, $(\tau_{fp})^{-1}$, as a function of the conformational barrier, where it is shown that the crossing time of the chemical barrier is independent of the characteristic time of motion along the conformational coordinate as long as $k_{conf} > k_{chem}$, and (**C**) τ_{fp} and τ'_{fp} (which is the fp time when we start the counting from the moment the trajectory reaches the RS). It can be seen that even when $k_{conf} < k_{chem}$, the time of crossing the chemical barrier is independent of the conformational landscape. (**B**) and (**C**) were originally presented in Ref. 67, and (**A**) was adapted in part from the same reference.

extreme possible effect of ligand binding) has no significant effect on the rate constant of the chemical step.

Obviously, we are not in any way claiming that the experimental studies discussed earlier are wrong, but merely that their dynamical interpretation is problematic. It could be argued that our simulation study[67] is the first to provide a molecular basis of the multitude of NMR and FRET observations, at least as far as coupling between the conformational changes and catalysis is concerned. On the basis of the data shown in Ref. 67 as well as the current available experimental and theoretical studies discussed earlier, we believe that it is unlikely that enzyme dynamics makes a significant contribution to catalysis.

As an aside, since the issue of semantics is being discussed at length in this article, it is worth mentioning studies such as that of Ref. 27 which bring up such terminology as "*the directionality of fluctuations*", or that of Ref. 28 which purports to "*show that pico- to nano-second timescale atomic fluctuations in hinge regions of adenylate kinase facilitate the large-scale, slower lid motions that produce a catalytically competent state*." The aforementioned study of adenylate kinase shows that this is in fact not the case. However, the more important issue is that whilst such statements are indeed quite elegant, it is not the beauty of the hypothesis, but rather the scientific facts backing it up which are important, otherwise such statements only serve to create even more confusion in a field that is already mired down in soft definitions and vague statements.

WHAT ABOUT MODE COUPLING?

Vibrational frequencies of catalytically important modes

It is tempting to suggest that special vibrational motions help in enzyme catalysis. An example of a study that might lead some to propose such an effect is a recent study of human aldose reductase[103] that uses a vibrational spectroscopy technique that measures the change in electric field at a specific site of a protein as shifts in frequency (Stark shifts) of a calibrated nitrile vibration. It is suggested that such shifts can be used to yield quantitative information on electric fields that can be directly compared with electrostatics calculations. To this end, the authors also perform molecular dynamics simulations in order to reproduce the observed changes in the field. However, at present, it would seem that only the vibrations of the probes in proteins were observed. That is, all other vibrations are implicit, as was, for instance, the case in resonance Raman studies of the photoisomerization of bacteriorhodopsin (e.g., Refs. 104–106). Thus, such studies have not actually elucidated (or even proposed) any relationship between special vibrational motions and catalysis, nor can these be coherently excited in thermal processes. Also, no relationship can be found between special vibrational motions and enzyme catalysis from simulation studies that have observed overdumped motions in proteins.[50]

Now, in the case of a proton/hydride transfer reaction, the donor-acceptor distance not only influences the height and width of the reaction barrier, but also affects the tunneling coefficient.[107] Thus, the rate of proton/hydride transfer is very sensitive to the donor-acceptor vibrational dynamics. Normal-mode analysis (NMA) provides a useful approach to study vibrational motions computationally.[108,109] Go and coworkers[110] have used this approach to evaluate the Franck-Condon factors for electron-transfer reactions of cytochrome *c*. Additionally, Cui and Karplus[111,112] have used an NMA to examine the projections of some of the modes of the protein-substrate system on the reaction coordinate in triosephosphate isomerase. They found that modes that symmetrically raise the energies of the reactant and product ("promoting modes") favor the reaction, while modes that affect the energies asymmetrically ("demoting modes") oppose it. Cui and Karplus[113] also found that crossing the barrier takes only about 30 fs for the proton-transfer step in triosephosphate isomerase, whereas the full redistribution of vibrational energy would take much longer. Therefore, they suggested that these nonequilibrium vibrational modes could influence the transmission coefficient through a dynamical effect (the problems with this assertion are clarified in Ref. 61). They nevertheless agree that any dynamical effect is likely to be minor, since the transmission coefficient is probably at least 0.5.

The limitation of NMA is that the harmonic approximation is not always valid due to the anharmonic nature of the proteins on one hand and the reaction on the other hand. However, it has been suggested in the aforementioned studies that NMA is an efficient way to acquire the intrinsic features of the collective motions of proteins, with qualitatively reasonable results. In addition, NMA consumes significantly less computational resources than MD simulations.

A general analysis of the coupling between the protein (or solvent) vibrations and the chemical process have been introduced by Warshel and coworkers in terms of the dispersed polaron (DP) spin boson treatment, which was first used in studies of electron transfer reactions and then in modeling enzymatic reactions.[25,63,114,115] This approach, which is based on the Fourier transform of the energy gap, provides the projections of the protein modes along the action coordinate and also allows one to explore nuclear quantum mechanical (NQM) effects in an approximated way.[25] This is achieved by relating the fluctuations of $\Delta\varepsilon_{12}$ during an MD trajectory to the fluctuations of an equivalent harmonic system. Here, we start with the autocorrelation function of the total energy gap:

$$C_i(\tau) = \langle u(t)u(t+\tau)\rangle \qquad (6)$$

where $u(t) = \Delta\varepsilon_{12}(t) - \langle\Delta\varepsilon_{12}\rangle$. The power spectrum of the fluctuations in a given diabatic state, $J(\omega)$, can be

obtained from the Fourier transform of the autocorrelation function:

$$J(\omega) = \left| \int_{-\infty}^{\infty} C(t) \exp(i\omega t) \mathrm{d}t \right| \quad (7)$$

$J(\omega)$ has peaks at the frequencies of the modes that are coupled to the reaction (ω_j), and in the high-temperature limit, the amplitudes of these peaks are proportional to the square of the displacement of the corresponding coordinate in ω_2 relative to ω_1:

$$J(\omega) = \pi\beta^{-1} \sum_j \hbar\omega_j \delta_j^2 \delta(\omega - \omega_j) \quad (8)$$

where index j now runs over the normal modes of both the solute and the solvent. The Fourier magnitudes obtained from Eq. (8) can be scaled by relating the area under the spectral density function to the overall reorganization energy (λ) as in Eq. (9):

$$\lambda = \frac{1}{2} \sum_j \hbar\omega_j \delta_j^2 = \frac{\beta}{2\pi} \left| \int_{-\infty}^{\infty} J(\omega) d\omega \right|. \quad (9)$$

Hynes[116,117] used a one-dimensional coordinate to describe the protein environment, and the promoting vibration was introduced to modulate the tunneling splitting. The studies on the rate-promoting motions were subsequently advanced by Schwartz and coworkers, whose analysis[118] suggested that promoting vibrations *only* play a role in the proton/hydride transfer if the frequency of the promoting vibrations is much lower than that of the barrier. Schwartz and coworkers used their approach in studies of several different systems[102,119,120] and, in all cases, argued for the importance of rate-promoting motions in catalysis. Unfortunately, *none* of these studies actually demonstrates that the promoting modes account for a catalytic effect (either by showing a rate enhancement relative to the reference reaction in water, or relative to different mutants). Other fundamental problems with this interpretation will also be discussed in greater detail in section "Networks of Correlated Motions and Related Issues."

Networks of correlated motions and related issues

Hammes-Schiffer and others[81,121,122] identified a network of correlated conformational changes with projections on the reaction path in simulations using the MDQT approach, but suggested that these reflect equilibrium structural effects rather than dynamical effects. QM/MM simulations described by Garcia-Vilocoa *et al.*[123] also appear to be in accord with this view. It is important to emphasize that in general, 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.[24,124] 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. The EVB and dispersed-polaron approaches consider the enzyme reorganization explicitly and automatically assess the complete structural changes along the reaction coordinates. As pointed out earlier, a dispersed-polaron analysis can for example provide information about the projection of the protein motion on the reaction coordinate and provides a basis for a quantitative comparison with a reference reaction in solution. Additionally, the coupling of protein motions to a reaction in an enzyme involves fluctuating electrostatic interactions of the solute with charged or polar residues and bound water molecules. In solution, it involves the reorientation of the solvation shells. Clearly, the reaction coordinate in both cases will involve components along the environmental (solvent) coordinate. The real difference is the amplitude of the change in the solvent coordinates during the reaction, which determines the reorganization energy and generally is smaller in the enzyme because of the preorganization of the active site.

Some of the misunderstandings with regards to the role of coupled motions may be associated with the implications that the chemical step in enzymes is facilitated by a network of coupled motions that bring the donor and acceptor to the correct distance and orientation and the correct electrostatic environment.[125] However, this interesting and appealing idea is still problematic, as the minimum of the free energy surface of the reaction in the folded enzyme (which determines the positions of the reacting fragments as well as the degree of electrostatic preorganization and the potential surface) also determines the nature of the reactive modes, which are in fact simply modes that have significant projections onto the reaction path. The reverse, however, is *not* true: that is, the reactive motions do not bring the system to the preorganized configuration but rather start from these configurations (at least in the native enzyme). Apparently, the folded enzyme establishes the free energy surface for the catalytic reaction, and the motions on this surface are merely a reflection of the Boltzmann probability of finding the system at different points on the surface. This point is illustrated in Fig. 7, and has been discussed in Refs. 126 and 127.

Schwartz and coworkers[120] have suggested that the catalytic reaction of purine nucleoside phoshorylase involves protein modes that reduce the barrier height by 20% (which is an enormous effect) by compressing the reacting fragments. To evaluate the contribution of such modes to catalysis, one must evaluate the barrier height from the minimum in the ground state, while taking into account the energy associated with the compression. Without such an analysis, one may find that a sufficiently strong compression would eliminate the barrier completely, resulting in a reduction of the barrier by 100%. Additionally, it is important to bear in mind that compression modes similar to those that occur in the protein also occur in the reference solution reaction. In the cases

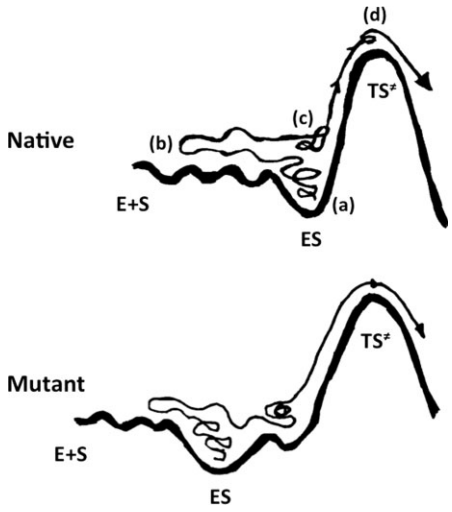

Figure 7

Highlighted here is the fact that motions to the ES region do not contribute to catalysis. That is, the figure illustrates that in a properly evolved native enzyme, the motions to and from the partially unfolded or unbound configurations are simply a part of the random excursion of the system around the ES minimum. Therefore, there are no fluctuations that "bring the system to the preorganized ES," as the system is already at the ES (where the fluctuations follow the Boltzmann probability, and the chance of reaching the TS region is determined solely by the free energy of the TS relative to the ES). The same thing holds for the mutants that were used to support the argument that the motion to the ES is relevant to catalysis. In fact, unless the motion to the ES is uphill (which means that the binding free energy is positive), k_{cat} is determined by the motion from the ES and not the motion to the ES [i.e., the reaction goes (a) → (b) → (c) → (d) and not (b) → (c) → (d)]. This figure was originally presented in Ref. 126.

that we have studied, the cost of bringing the reactants to the same distance are similar in solution and in the enzyme, which means that the compression does not contribute significantly to catalysis (see the related discussion of near-attack conformations in Refs. 128 and 129). Thus, the problem is that there is simply no attempt to determine the rate enhancement (or barrier reduction relative to any reference) by the enzyme, which is essential when attempting to explore catalytic effects. Furthermore, there is no attempt to reproduce the mutational effect on the barrier. In fact, there is no attempt to calculate or formulate the magnitude of the rate enhancement associated with the dynamical effect of the protein-promoting vibrations. In particular, while the study of Ref. 120 presents the difference in the power spectra of the H257G and H257A mutants, it has not provided the corresponding difference in activation free energy ($\Delta g^{\neq}$) between theses mutants. Claiming that there is a change in the projections of different normal models upon mutation does not provide any evidence of a dynamical effect since this is simply the trivial result of the change of the potential energy surface. Here, one must define what the dynamical effect is, and then convert it to a change in rate constant. Not doing so may amount to semantic excess and potentially to a misleading message to these who confuse such studies with a proper analysis of a catalytic effect where the proposed effect is actually calculated. This might also reflect the belief in some circles that no enzymatic rate can be actually calculated, and thus simulating changes in some other property is equivalent to simulating the assumed resulting changes in the rate constant.

Flexibility and catalysis

It has been frequently implied that flexibility helps enzyme catalysis (e.g., Refs. 6, 130–134 amongst others), and this seemingly appealing idea requires some critical examination. That is, the idea that motions are needed for catalysis could, in principle, yield the conclusion that the catalytic power of enzymes is connected to their flexibility.[135] In this vein, several workers have used studies of the thermal adaptation of enzymes to argue for dynamical contributions to catalysis.[6,130–132] More specifically, it has been argued that thermophilic (Tm) enzymes (that have evolved to function at highly elevated temperatures) should at most temperatures be more stable than the corresponding mesophilic (Ms) enzymes (that have evolved to function at room temperatures). It is known that Tm enzymes have lower catalytic power than Ms enzymes at the same low temperature, and, based on this, it has been argued that the low catalytic power is presumably due to a decrease in dynamical motion.[130,136,137] The possible relationship between the thermal stability and catalytic power of enzymes (and the related implications that the reduced dynamics of Tm enzymes is the root cause for their reduced catalytic power) was recently examined in the specific case of DHFR by means of detailed simulations.[138] This study found that while the Tm enzymes do indeed have restricted motions in the direction of the folding coordinate, this is not relevant to the chemistry of these enzymes, as the motions along the reaction coordinate are *perpendicular* to the folding coordinate. Additionally, this study demonstrated that the rate of the chemical reaction is *not* determined by dynamics or flexibility in the ground state, but rather, it is determined by the activation barrier, which is in turned determined by the corresponding reorganization energy. Furthermore, it was found that the displacement along the reaction coordinate is in fact larger in the case of the Tm enzyme than in the case of the Ms, which is the opposite of the trend along the folding coordinate (and is of course contradictory to the flexibility proposal). This is consistent with the idea that the general trend in enzyme catalysis is that the best catalyst requires less motion during the reaction than other less optimal catalysis, and that to obtain a small electrostatic reorganization energy, some of the

folding energy has to be invested into the overall preorganization process, leading to less stable optimal catalysts.

FREE ENERGY LANDSCAPES AND CATALYSIS

The complexity of the free energy protein landscape of proteins and other considerations led some to suggestions that this concept can help to rationalize the catalytic power of enzymes,[95,139–142] such as, for instance, Boehr and coworkers[71] who stated that "*the modulation of the energy landscape by the bound ligands funnels the enzyme through its reaction cycle along a preferred kinetic path*," or Benkovic and coworkers[139] who argue that "*the free-energy description of enzyme catalysis cannot be described in two dimensions but requires a multidimensional free-energy landscape that is very rugged with multiple minima and transition states. Thus, enzyme reactions can be regarded as operating through 'catalytic networks' to achieve their remarkable efficiency.*"

Now we completely agree that the folding landscape is an important concept in describing the dimensionality of proteins (an issue that presents a significant challenge for simulation approaches), and we have discussed landscape effects before elsewhere.[37,98,100,143] However, the landscape can be described clearly in two dimensions, since a two-dimensional description is still far better than *no* dimensions (which is what many workers do in their discussions), or just one dimension. In fact, a two-dimensional description is the most effective way to analyze the chemical and conformational landscape (it is also what is done in folding studies), since we cannot visualize higher dimensionality. The trick, however, is to be able to project all the physics of the many dimensional system onto the two-dimensional space (see below). Furthermore, we do not see it as any source of catalytic effects in itself, but rather as just a way to define the relationship between the chemical and conformational coordinates. This point will be discussed in more detail below.

This issue of the catalytic landscape has been highlighted by recent experimental work on chorismate mutase.[144,145] Here, Hilvert and coworkers utilized NMR and various other biochemical techniques to study a highly active monomeric chorismate mutase that was obtained by the topological redesign of a dimeric helical bundle enzyme from *Methanococcus jannaschii*. This enzyme is key to catalyzing the conversion of chorismate to prephenate (see Ref. 146) in the biosynethsis of 1-tyrosine and 1-phenylalanine into a highly active monomer (mMjCM). Interestingly, unlike its natural counterpart, the monomer unexpectedly possesses all the characteristics of a molten globule but still works as well as the native enzyme. These observations seem to challenge the conventional view that efficient catalysis requires an exquisitely preorganized active site structure.

Thus, we recently explored[100] the relationship between the folding landscape and catalysis in CM by a simulation study. This study evaluated the chemical activation barriers for different regions of the protein conformational coordinate. It was found that the CM monomer (that behaves like a molten globule in the absence of the substrate) has low activation barriers even in regions that are not exactly at the native configuration (see Fig. 8).

Xiang *et al.*[143] have also performed theoretical studies of the relationship between the conformational landscape and catalysis, in the case of the enzyme DNA polymerase β. The aim of this study was to attempt to elucidate how this enzyme discriminates between incorrect (W) and correct (R) nucleotide incorporations by examining the conformational landscape for this enzyme, and the simulations indicated that the transition states for the incorporation of R and W nucleotides reside in substantially different protein conformations. This work introduced structure-based calculations of the catalytic landscape, and also formulated some of the relevant questions in terms of the shape of the landscape, such as the possible effect of reactive trajectories in different protein configurations.

The question about the role of the landscape in catalysis was addressed in a recent study[139] (which was mentioned at the beginning of this section), where it was argued that the enzyme mechanism should be viewed as "catalytic networks," in which multiple conformations occur during the mechanism (both serially and in parallel), and then form coupled ensembles of conformations that require a "rugged" multidimensional free-energy surface with several minima and transition states. In fact, this point was first formulated in our work[143] (see Fig. 9), however, some issues about the impact of this effect on the rate remain unresolved (see the discussion below).

It is most useful to return here to the adenylate kinase system that was discussed extensively in "The Experimental Search for the Dynamical Coupling Between the Conformational and Chemical Coordinates" Section, as mentioned earlier, we have recently modeled the landscape and dynamics of this system on the millisecond timescale.[67] Our studies (and in particular those with the full CG model) demonstrate that the landscape is not smooth. What remains to be done now is to quantify the barriers between the different valleys that lead to the TS, and to actually calculate the effect of having several passages in the limit of the low and high barriers. At present, we do not see any reason why the inclusion of this effect will drastically change the rate constant.

The apparent complexity of the landscape concepts may be a reflection of the fact that some people have difficulty with visualizing multidimensional details, as they are distracted by the fine details, and do not have experi-

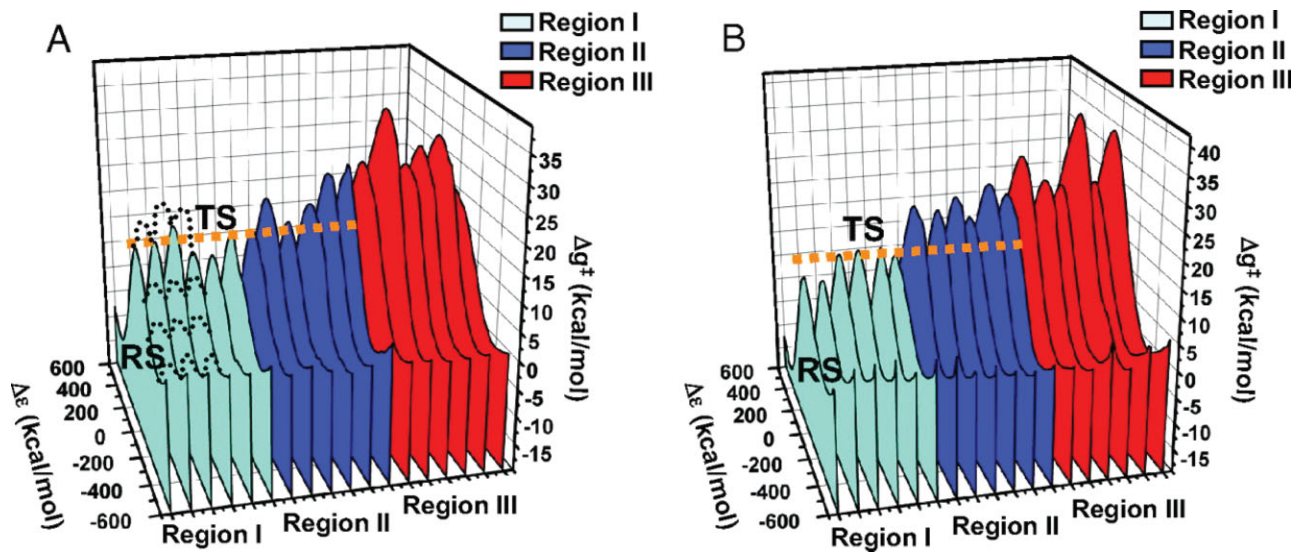

Figure 8

The landscapes for the chemical profiles for the monomeric (**A**) and dimeric (**B**) forms of chorismate mutase. Here, the profiles are equally spaced according to the rmsd from the native structure for the three regions (I, II, and III) of the enzyme. The orange dashed line designates the 16 kcal/mol height that corresponds to a reasonably low activation barrier, from which it can be seen that the monomer has several catalytic configurations in the second region, whereas the dimer does not have any. This figure was originally presented in Ref. 100.

ence with the fact that computers can actually handle these details quite well, and convert them to lower dimensionality models. This problem is probably one of the factors contributing to the confusion that exists about the preorganization effect[147–150] (see "Enzyme Catalysis Is Due to Polar Preorganization, But This Has Little to Do With Recent Dynamical Definitions of Preorganization and Reorganization" Section, and to the proposal that searching the landscape leads to or accounts for the catalytic effect.[151] This is an extremely problematic proposal, as searching the landscape is the normal behavior of any system as it moves with the Boltzmann probability, and what is *actually* important is the height of the free energy barrier. When we have several passes on the TS ridge (see Fig. 9), we have to take them into account by considering the activation entropy, but the same treatment must also be done on the ground state. The net effect is thus small and conceptually trivial, and of course does not explain catalysis.

In our opinion, the only way that the landscape can contribute to catalysis is by having a much larger configurational space at the TS (which is perhaps what Klinman vaguely referred to as a funnel[149] or what Nussinov[142] had in mind). However, no such funnel is found when one explores the actual landscape in real enzymes (see Fig. 9). More specifically, a prominent example of the earlier issue is provided by the recent entropy funnel model presented in e.g. Ref. 149 (see Fig. 9 of Ref. 149). This model (which is quite ill defined and is brought here as a key illustration of the need for a clear physical description) takes the frame of reference as being the top of the funnel, which is defined as a 100% probability for any protein conformer to achieve one of the many catalytically relevant interactions, which can be formed between either different substrates with each other or the protein. It was suggested that moving down the funnel

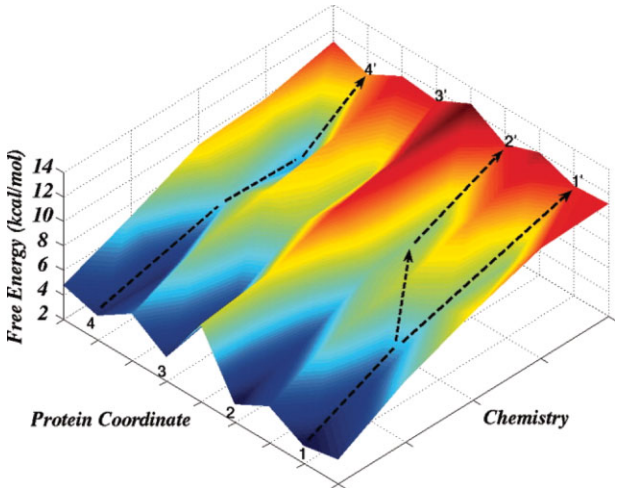

Figure 9

A schematic representation of the free energy landscape as a function of the conformational and chemical coordinates in a reacting enzyme. The figure depicts trajectories across the conformational coordinate and a continuation of this trajectory along the chemical reaction coordinate. This figure is adopted in part from Ref. 143.

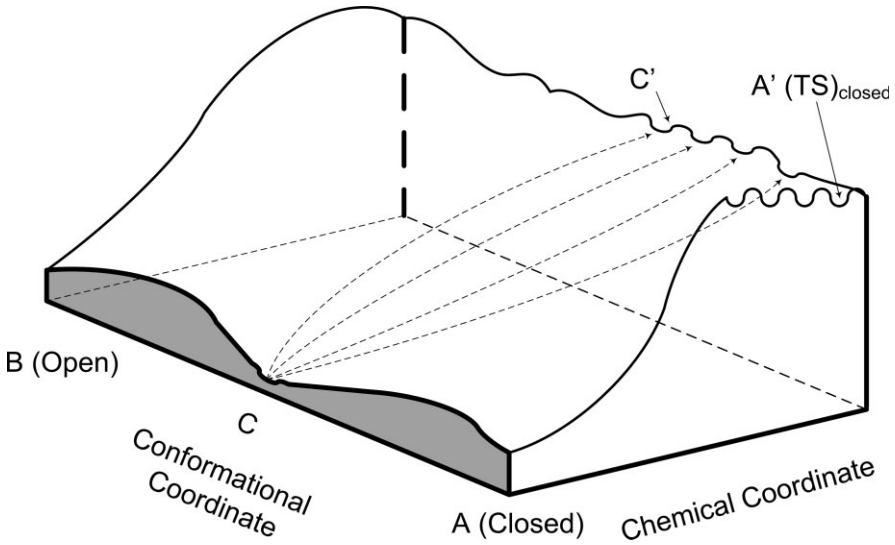

Figure 10

Examining the relationship between the free energy landscape and entropic effects. This figure serves to illustrate the fact that the recent suggestion of the existence of an "entropy funnel"[149] has no relationship to the actual free energy landscape.

then progressively decreases the probability of finding conformers with increasing numbers of substrate/protein interactions, until the family of conformers with sufficient numbers of interactions for catalysis to proceed is reached. Thus, progression along the funnel (from top to bottom) is supposed to represent an overall decrease in entropy. This model is then used to try to account for the $T\Delta S^{\ddagger}$ temperature dependence of thermophilic ADH (see the next section for further discussion of this issue). That is, the "freezing out" of protein flexibility, which is supposed to accompany a reduction in temperature for the thermophilic proteins, is argued[149] to be representative of a more restrictive conformational space, occurring further down the funnel, where it becomes necessary to increase protein disorder in such a way that the protein moves into the range required for optimal catalytic conditions. Unfortunately, this seemingly detailed proposal (which is in fact as vague as many of the dynamical proposals discussed earlier) mixes undefined "catalytic conditions," and thus also presumably the catalytic coordinate, with some elusive protein coordinate. To start with clarifying the problems, we would like to point out that a surface is not described by forming different interactions, but rather by the effect of all the interactions on the potential surface along a given reaction coordinate. Unless this proposal is formulated in clear physical terms that can be thoroughly explored (like the proposal being considered in Fig. 10), it cannot really be considered a proposal, and even the nature of the proposed funnel has no relationship to the clear landscape description, or to any conceivable description of physical landscapes. In other words, once we try to describe the entropic funnel idea we *must* select one of the options presented in Fig. 10, and this is done below.

The first option is related to the entropic effect in the ES region (Point A of Fig. 10). In this case, the proposal implies a large entropic contribution to k_{cat}, whereas in most cases, this contribution is actually very small.[25,152] Furthermore, in this case it does not matter if the entropic effect comes from the nearby protein groups[25,37] or from the relaxation of the whole protein (see Åqvist's analysis of psychrophilic enzymes[153] for an example of this), as it occurs in the closed configuration and has little to do with the implications of the funnel proposal. Of course, the origin of activation entropies in enzymes has to be analyzed computationally, as was done by our restraint release approach in a related case.[154]

The second possibility is that the proposal implies that the reactive trajectories can pass through many points at the TS (moving from C to C' of Fig. 10), starting from a restricted ground state region. Unfortunately, this proposal is inconsistent with any modeling study.[100,143] That is, of course the landscape can be very complex (though contrary to the claims of Ref. 142 this is not necessarily a fundamental problem, nor does it on the other hand provide a catalytic advantage), and this complexity can in principle include a scenario where the TS region has more configurations than the RS. However, as the activation entropies are not large in real enzymatic reactions,[25,152] it is highly unlikely that such a scenario can occur, and, even in a scenario with a heterogenous set of barriers (similar to that observed in Ref. 100), the average rate is still determined by the highest barriers for the chemical step (which is in turn determined by the

corresponding reorganization energy), as long as the barriers between the different configurations in the ground state are lower than the chemical barrier, the solution of the multistate rate equation will simply follow the trend dictated by the highest activation barrier(s).

The final possibility is that somehow the landscape is very narrow at point B, and then we have a large entropic effect upon moving to point A. This is simply a case of a binding entropy effect, which is not observed experimentally, and, more importantly, has no effect at all on the chemical step (i.e., the binding free energy has no effect on the chemical barrier, which reflects the differential binding of the TS). It seems to us that the proposal implies a configurational search on the way to the TS, but it cannot define how this can actually be done. Here one of the major problems is that the activation entropy simply does not reflect any such search, but rather, being a state function, it is simply the difference between the entropies in the initial and final state.

Overall, we like to point out that unless the entropic funnel proposal is described in terms of some landscape diagram it has no well defined meaning and cannot be explored or exclude in a scientific way. However, while trying to find the most reasonable definitions, we can show that at least with such a definition, the proposal is not likely to account for catalysis.

TUNNELING AND OTHER NUCLEAR QUANTUM MECHANICAL EFFECTS

Enormous information about biological mechanisms has been obtained by studies of isotope effects in enzymatic reactions.[155,156] Such studies also provided excited information about nuclear tunneling in enzymatic reactions (e.g., Refs. 151, 157–159). However, the finding of nuclear quantum mechanical effects (NQM) has also led to proposals that are strongly related to the dynamical proposal. This has had several major implications including the suggestions that the tunneling correction presents a major deviation from TST and that it provides a major catalytic advantage, and that the temperature dependence of the isotope effects implies special catalytic dynamical effects. We will show below that both implications are neither justified nor supported by any unique experimental or theoretical study.

NQM effects *do* occur in enzymes, but the same effects also occur in the corresponding solution reactions

Studies of isotope effects in some enzymatic reactions have pointed to nuclear tunneling and other nuclear quantum mechanical effects, such as the zero-point energy contributions of the participating vibrational modes.[160] For example, Klinman and coworkers have provided clear evidences for nuclear tunneling in alcohol dehydrogenases,[6,161,162] serum amine oxidase,[163] lipoxygenase,[164] and glucose oxidase.[165] These findings have frequently been interpreted as evidence for dynamical effects. That is, it has been suggested that an enzyme may, for example, exploit a particular vibrational mode that modulates the thickness of the barrier through which an atom can tunnel. The argument that NQM effects contribute to enzyme catalysis is based on the observation of large isotope effects in enzymatic reactions as well as other indications of nuclear tunneling (e.g., Refs. 160, 166). These findings are both very reasonable and very important. They are also fairly consistent with the existence of nuclear tunneling, and are reproducible by simulation studies.[167,168] The problem is, however, that such contributions are not necessarily catalytic, since they *must* be quantified relative to the corresponding reference solution reactions. However, as we have already pointed out in several of our papers,[37,96,167,169,170] the same NQM effects that occur in the enzyme also occur in the reference reaction in solution, and contribute in a similar way.

Similar findings have also been made in the several experimental studies in which the reference reaction was experimentally observed (such as in the key work of Finke and coworkers[171]). We should nevertheless point out that in many cases it can be hard to measure the relevant reference reaction, and computer simulations thus offer a reasonable way to explore the reference reaction[172–176] and the corresponding NQM.[168,169] However, such studies are only meaningful if they can reproduce the observed catalytic effects, and, fortunately, our EVB approach (which is calibrated on *ab initio* solution studies) provides the needed reliability. Now, EVB studies coupled with quantum classical path (QCP) calculations[168] of isotope effects have reproduced both the observed catalysis as well as the observed isotope effects in any systems studied by us. The same calculations also found that the isotope effects are similar in the enzymatic and solution reactions.[169]

The most basic argument about the role of tunneling in enzyme catalysis starts with the idea that the enzyme compresses the distance between the donor and acceptor, thus leading to a narrower potential and to greater tunneling.[162,166,177–180] This idea, that now appears to be taught in standard undergraduate biochemistry courses, is very appealing. It has also been used to rationalize the temperature dependence of observed KIEs.[179] However, both us and others have recently found[168,181,182] that in fact even in the vibronic formulation, the isotope effect *increases* due to the sharp distance dependence of the zero–zero vibrational overlap (see Ref. 126 for a clear analysis). That is, in the more recent view, the NQM effects *decrease* rather than increase upon compression. This effect is due to the fact that the tunneling in proton and hydride transfer reactions depends on the overlap between the vibrational wave functions of the reactant and product

states, and this overlap in turn depends on the distance between the corresponding minima (see Ref. 168). In fact, when the donor and acceptor are pushed to a short enough distance, the mixing between the two states makes the adiabatic surface very flat, and the tunneling effect disappears. Although the aforementioned claims may sound strange to some, the simplest way to convince the reader is to point out that all key workers in this field (e.g., Refs. 181 and 182) now essentially obtain this result.

The aforementioned analysis has, of course, major implications with regards to the idea that NQM effects make a significant contribution to enzyme catalysis. In fact, the effects that lead to an increase in the NQM contributions appear to be anticatalytic. This is mainly due to the fact that the rate constant is smaller for larger donor–acceptor distances. Because of the fact that the observation of a large KIE reflects an increase (rather than decrease) in the distance between the donor and acceptor is a seemingly counterintuitive point, it still causes significant confusion (see e.g., Ref. 126 for further discussion of this issue).

The temperature dependence of isotope effects does not provide support to the dynamical idea

The temperature dependence of the NQM effects as manifested in the corresponding KIE has been a topic of significant current interest.[160,167,181,183] Part of this interest stems from the hope that this temperature dependence can provide useful information about possible dynamical contributions to enzymatic reactions.[160,184]

As far as tunneling is concerned, there is a tendency to argue that the observed temperature dependence of KIEs (e.g., Refs. 185 and 186) is clear evidence for thermally activated tunneling, and thus, this can be considered support for the idea that tunneling contributes to catalysis. We have no problems with agreeing that tunneling in enzymes can be thermally activated. In fact, our simulations and formulations have long been consistent with this view.[37,96,167,169,170] We have also recently succeeded in reproducing the observed temperature effect.[168] However, our simulations have led to the conclusion that NQM effects do not help in catalysis, since the same thermally activated tunneling also exists in solution.

With the above findings in mind, we can ask what the actual meaning of the observed temperature effect is, and what it tells us about catalysis. Apparently, the studies of DHFR[168] and lipoxygenase[167] indicate that the KIE increases when the distance between the donor and acceptor increases (see the previous section). At any rate, the temperature dependence of the KIE appears to mainly reflect the temperature dependence of the distance between the donor and acceptor. Thus, the temperature dependence actually indicates that tunneling is anti-catalytic (see also our recent review[126]).

Kohen *et al.*[6] made the interesting observation that the activation enthalpy ($\Delta H^{\ddagger}$) for the reaction of the thermophilic alcohol dehydrogenase decreased from 23.6 kcal/mol at low temperatures (0–30°C) to 14.6 kcal/mol at higher temperatures (30–65°C). They interpreted this observation as supporting a contribution to k_{cat} from vibrationally enhanced tunneling at higher temperatures. The activation free energy, however, remained essentially constant. In terms of TST, this means that a compensating increase in $-T\Delta S^{\ddagger}$ accompanies the decrease in $\Delta H^{\ddagger}$ as the temperature is raised.

Although Kohen *et al.*[6] attributed their finding the dynamical effects, in that they believed that their findings on hydrogen transfer under physiological conditions could not "be explained without invoking both quantum mechanics and enzyme dynamics," Warshel and coworkers have noted (e.g., Refs. 25, 61) that the entire effect is in fact a simple entropic effect, and the observed decrease in $\Delta S^{\neq}$ with temperature in the alcohol-dehydrogenase (ADH) reaction can be rationalized by considering the expected interactions of the solute with its surroundings. As the reaction in the direction considered by Kohen *et al.*[6] proceeds from a polar ion-pair through to a less polar transition state to a nonpolar product, the motions of the surroundings are expected to be less restricted in the transition state than in the reactant state, which contributes a positive term to $\Delta S^{\ddagger}$. Raising the temperature will release some of the motions that are frozen in the reactant state, which should make $\Delta S^{\ddagger}$ less positive.

It should be noted that even some of the workers who originally supported the dynamical explanation *now* agree with the importance of entropic effects,[149] though this is attributed to the new concept of a funnel effects (see Ref. 149, though this work attributes this to the new concept of a funnel, as discussed in section "Free Energy Landscapes and Catalysis"). This presentation is then used to account for the $T\Delta S^{\ddagger}$ temperature dependence of thermophilic ADH (which was one of the central issues of our previous analysis). This "freezing out" of protein flexibility, which is supposed to accompany a reduction in temperature for the thermophilic proteins, is argued[149] to be representative of a more restrictive conformational space, occurring further down the funnel, where it becomes necessary to increase protein disorder in such a way that the protein moves into the range required for optimal catalytic conditions. As discussed in section "Free Energy Landscapes and Catalysis," such a proposal has not yet been (and probably cannot be) formulated in such a way that can be analyzed, and our attempt to define such proposal resulted in models that are not likely to be supported by any calculations or conceptual considerations. The proposal also overlooks that fact that in most enzymatic reactions, $-T\Delta S^{\ddagger}_{cat}$ is very close to zero (see Refs. 25, 152). Furthermore, this has little to do with catalysis, since $\Delta G^{\neq}$ is practically constant and temperature independent in the cases being considered. Finally, even if in some unclear way it is be found by means of calculations that the entropic effect in

ADH is due to some changes occurring far from the active site for case A in Fig. 10 (which is far less likely than the simple local electrostatic idea that was proposed by us[25]), it will have no dynamical implications since it is a simple issue of the configurational space. We must clarify here that case A in Fig. 10 probably has *nothing* to do with the entropy funnel concept. In fact, our recent calculations (in preparation) did reproduce the observed effect, thus confirming our previous analysis.

"High percentage tunneling" and other considerations

At this point we like to comment on the fact that there have been some recent studies which have classified reactions with a relatively small tunneling contribution[187–189] (i.e., a reduction of 4% in the barriers) as proceeding predominantly by tunneling. This view is based on the fact that the NQM contribution to the rate constant is more than 50% (in one case, it is even claimed that the reaction only proceeds 1% by the classical way, i.e., 99% by tunneling[188]). This classification seems to reflect a major misunderstanding, and is actually misleading. This point is discussed clearly in Ref. 126 but here we will clarify that the reaction barrier changes only from e.g., 15 to 14 kcal/mol[187] when we add tunneling and zero point energies, were the 1 kcal/mol is trivial as compared to the actual classical effect of 15 kcal/mol. Perhaps the best way to see the falacy of the aforementioned definition is to realize that in order to distinguish between a classical mechanism with proceeds over the barrier to a tunneling mechanism which proceeds through the barrier, one must be able to quantify what fraction of the molecules are reacting via tunneling, and how many are reacting classically. We do not believe that there is any logical way to relate a trivial reduction in a rate constant by a factor of $\sim$20 to a change in the reaction from 100% of the molecules reacting classically to a situation where 95% are reacting through tunneling (i.e., the only point that was established in the aforementioned works is that the *rate* changes, not that the fraction of molecules tunneling through the barrier changes by that percentage). Of course there are ways to actually calculate the fraction of trajectories that react classically, and those that tunnel through the barrier. However, no such approach was used in the above definition, which confuses the percentage tunneling with the percentage change in the rate constant, and tries to convert a trivial effect into an enormous factor.

In summary, a careful analysis of the available experimental studies concluded that there exists *no single piece* of consistent experimental evidence that demonstrates that tunneling makes a significant contribution to catalysis, whereas all computational studies that actually explore this issue have consistently have found that the catalytic effect of NQM is very small (if there is any at all). Once again, we refer the reader to our recent review[126] for further discussion.

WHAT ABOUT CONFLICTING THEORETICAL FINDINGS?

At this point in the review, it is important to address any potential concerns that the reader may have that perhaps our perspective is too biased, and that somehow other theoretical studies have reached different conclusions. This issue has already been partially addressed earlier in this work. However, it is useful to focus here on the findings of the key studies that have explicitly or implicitly supported the dynamical idea. In particular, we would like to convey the point that theoretical studies that are supposed to determine catalytic contributions *must* be able to actually calculate the given catalytic effect so that the effect is clearly estimated. Such studies must also be able to evaluate the overall catalytic effect reasonably quantitatively, so that the there is a way to assess the quantitative level of the given estimate.

Some of these issues were considered systematically in previous works,[25,61] and, in this review, due to space limitations, we have only provided a few instructive examples (e.g., the discussion of Ref. 120 in section "Networks of Correlated Motions and Related Issues" and also in the discussion of Ref. 190 that is presented in Ref. 37) in order to try to give the readers a few pointers on how to evaluate theoretical studies of dynamical effects (such that even if the reader does not necessarily agree with our conclusions, at least they are aware of what has actually been established through theory). Here, we would like to highlight only one particular example (namely Ref. 158) of such problematic theoretical studies. We will use it to demonstrate the point that many theoretical studies along this line are forced to mislead the reader about the background and conclusions of the work (in order to support the dynamical and related proposals), and/or to present an incorrect analysis. We will do this in significant detail, not as a retroactive referee report, but rather as a general example of the problems with such works. At any rate, Ref. 158 attempts to feature the results of a decent calculation (using basically our QCP approach[191]), which obtained a minor NQM catalytic contribution, as major support for the completely opposite idea of a catalytic tunneling effect. This is done by examining the quantum tunneling contributions in the proton transfer reaction catalyzed by nitroalkane oxidase, and its counterpart in solution, and purporting to find a transmission coefficient that is approximately three times greater in the case of the enzymatic reaction than in solution. Unfortunately, the work is flawed on many points. The first is the misrepresentation of the literature on almost all fronts—this starts by presenting the authors of Ref. 158 as the main proponents of the idea that

enzyme catalysis should be examined in comparison to the reference in solution (while we have been the only group studying this issue consistently since 1978, as can be seen from Ref. 37 and references therein). Similarly, the authors chose to neglect the works that championed the importance of comparing tunneling in the enzyme and solution (and introduced such studies), such as for instance, Refs. 115 and 169. Finally, the authors argue that the approach they are using is unique in being able to yield accurate KIEs, not mentioning the fact that their computational method is in fact our QCP approach, which was introduced by us and already successfully used to study the same problems in several systems (e.g. Refs. 168 and 169). This problem, which is compounded by attributing this approach to those who never introduced it (e.g. Ref. 25 of Ref. 158), does not instill confidence in the authors' scientific analysis.

However, more serious than this are the scientific problems with the work. The first is that the authors are presenting a very trivial effect—i.e. the overall classical barrier reduction and the NQM reduction are about 8 kcal/mol and 0.4 kcal/mol respectively. Second, the fact that NQM contributions to catalysis are small but might in some cases not be zero, was already found, analyzed and discussed by us in Ref. 169. Third, the authors persist in propagating the idea that the enzyme has evolved to make the proton transfer barrier narrower, thus enhancing tunneling. Now this is extremely problematic (see section NQM Effects Do Occur in Enzymes, But the Same Effects Also Occur in the Corresponding Solution Reactions). That is, as was discussed in, amongst other works Refs. 126 and 127, the KIE in fact *increases* when the DAD decreases, and thus, the increase in the relative NQM contributions *must* reflect an increase (rather than decrease) in the optimal DAD distance, which reduces the classical catalysis.

Furthermore, Fig. 2 of Ref. 158 (which depicts the computed classical and quantum potentials of mean force for the reaction in the enzyme and in solution) shows that at the TS, the surface in solution is *not* wider than that in the enzyme. Note also that the width at the base is meaningless since it includes a large classical solvent reorganization contribution, which has been found in serious studies (such as that of Ref. 96) to only make a minor contribution to the NQM effect. Also, the effective potential shown in Fig. 4 of Ref. 158 is quite misleading. First, the figure is not obtained by the QCP, or the proper classical sampling used in Fig. 2 of Ref. 158, but rather by the VTST approach, which does not include sufficient sampling, and gives very different results than those actually obtained by the quantized PMF of Ref. 158. The sections of the potential surface in water include some with enormous width, which are basically irrelevant, and probably represent high-energy regions that do not contribute to the rate. These sections were probably taken without correctly considering the absolute energy and the penalty of going to those TS regions (see Ref. 168 for a proper analysis). Perhaps the main problem here is associated with taking the sections, while starting from the TS, with a *fixed* protein configuration, instead of determining the height relative to an absolute minimum. Overall the problems with Fig. 4 are obvious, as they contradict the actual quantized PMF, which was accurately evaluated in Fig. 2 (if these regions were relevant, we could never obtain identical width for the enzyme and water as shown in Fig. 2). There is, in fact, no problem with getting somewhat larger NQM in the enzyme, as we already showed in 1996,[169] and this might be a result of the classical barrier reduction (see discussion in Ref. 169). However, since all reliable calculations (including those with the QCP, as demonstrated in Ref. 96) now show that the KIE *decreases* rather than increases with compression, the argument that the NQM is increased due to barrier compression is extremely problematic. It would have been instructive to see the authors of Ref. 158 reporting the result of increasing the DAD distance in the enzyme.

It is not for us to judge whether the practice of taking results that actually contradict the tunneling or dynamical idea and present it as support for the idea is more problematic than the practice of reaching the same conclusion by incorrect and/or irrelevant calculations (that give completely incorrect catalytic effects, as was done by Schwartz and coworkers[102,118–120] and discussed in part in the section "What About Mode Coupling?"). However, we would like to guide the reader to the need for critical examination of theoretical studies that seem to support the dynamical idea, as none have emerged up to now, and the pressure of getting theoretical support for the problematic dynamical idea seems to lead to uncritical studies. Thus, we believe that the aforementioned examples (including the one discussed above), as well as independent critical judgment, will lead the reader to agree that while there have been significant advances in theoretical studies of this question, there still exists *no* single consistent study that has actually demonstrated that dynamical effects significantly increase the rate constant.

ENZYME CATALYSIS IS DUE TO POLAR PREORGANIZATION, BUT THIS HAS LITTLE TO DO WITH RECENT DYNAMICAL DEFINITIONS OF PREORGANIZATION AND REORGANIZATION

The issues presented in this section have to some extent already been discussed in Ref. 37. However, here we would like to clarify some key issues about the preorganization effect. That is, as demonstrated by many of our works (see the summary in Ref. 37), the electrostatic preorganization effect has been found to account for

most of the catalytic power of any enzyme we have studied. However, experiments (such as for instance that of Ref. 192) which explored the role of electrostatic effects (by the use of charged groups in order to stabilize the TS charge distribution) came to the conclusion that such effects must necessarily be small. Phenomenological attempts to estimate the magnitude of electrostatic contributions[193] to catalysis reached similar conclusions. Thus, it was considered more or less "common knowledge" that electrostatic effects *do not* play an important role in catalysis (even though physical organic chemistry experiments in solution could be rather irrelevant to an active site, and phenomenological attempts to estimate the strength of electrostatic effects in proteins simply cannot assess the dielectric effects without a proper computational model). Similarly, in his key work, Jencks[194] did not consider electrostatic stabilization of the TS as being important for catalysis (which is in clear contrast to recent suggestions, such as those of Ref. 148), but rather argued that electrostatic and desolvation effects are the price to be paid for substrate "destabilization." Thus, it was not until the 1976 work of Warshel and Levitt[195] that anyone really demonstrated that electrostatic effects can play a major role in enzyme catalysis. We should point out that per definition,[40,195,196] "electrostatic catalysis" includes the effect of the protein charges, permanent dipoles (residual charges), induced dipoles (polarizability), and solvation by bound water molecules. It does not, however, include the van der Waals strain effects, orientational entropy, charge transfer covalent interactions, or dynamical effects. However, since 1976, there has been growing theoretical support for the role of electrostatic stabilization in catalysis (e.g., Refs. 26, 63, 128, 197–199 amongst others), and in fact it is becoming quite clear that electrostatic effects are central to catalysis (for a detailed review, see Ref. 37).

As shown in Ref. 37 there are multiple cases where most of the catalytic effect is clearly due to electrostatic interactions, though it is of course important to be able to establish that these effects are due to TS stabilization, and to determine why the protein can provide such large effects. This issue can be explored using the LRA expression below[200] for both the TS and RS:

$$\begin{aligned}\Delta G(Q^{\neq}) &= 0.5(\langle U(Q=Q^{\neq}) - U(Q=0)\rangle_{Q=Q^{\neq}} \\ &\quad + \langle U(Q=Q^{\neq}) - U(Q=0)\rangle_{Q=0} \\ &= 0.5(\langle \Delta U\rangle_{Q^{\neq}} + \langle \Delta U\rangle_{0}) \end{aligned} \tag{10a}$$

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

where U is the solute-solvent interaction potential, Q designations the solute residual charges, $Q^{\neq}$ indicates 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 λ in Eq. (10b) denotes the reorganization energy for the solvation of the TS, which will be discussed below. The first term in Eq. (10) is the interaction energy at the TS, where $Q = Q^{\neq}$, and this is similar in enzymes and in solution. The second term expresses the effect of the preorganization of the environment: if this is randomly oriented towards the TS in the absence of charge (as in the case in water), then this second term is zero, and we get:

$$\Delta G(Q^{\neq})^{w}_{sol} \cong \frac{1}{2}\langle \Delta U\rangle_{Q^{\neq}} \tag{11}$$

Here, the electrostatic free energy is half of the average electrostatic potential.[201] However, this does not hold in the preorganized environment of the enzyme, where we have a significant contribution from the second term, giving an overall $\langle \Delta U\rangle_0$ that is more negative than in water. This is a result of the catalytic effect of the enzyme. Another way of seeing this is to picture the fact that in water, the solvent dipoles are randomly oriented around the uncharged form of the TS, and the activation free energy includes the free energy that is required to reorganize these solvent dipoles towards the charged TS. On the other hand, in protein, this reaction costs less reorganization energy, as the active dipoles (which come from polar groups, charged groups and water molecules) are already partially associated towards the TS charge.[202] This concept is illustrated in Fig. 11. This effect is related to Marcus' well-known reorganization energy, however, it is not identical to it, as Marcus' reorganization energy[204] relates to transfer from the reactant to product state, whereas we are dealing here with charging the TS. The conceptual and practical differences between the two have been discussed in detail in Refs. 20 and 37.

Although the preorganization idea has been repeatedly confirmed by consistent simulation studies it has not been fully accepted, partially because it is not simple to understand this concept intuitively, and thus to realize that the catalytic effect is *not* due to the enzyme substrate interaction, but rather to the energy change (i.e., the small reorganization in the preorganized enzyme) in the enzyme. This might be one of the reasons for the constant search for other catalytic effects, such as the dynamical proposal. Additionally, it is not helpful when other workers keep redefining the concept of preorganization, for instance in the case of Schlick *et al.*,[150] who incorrectly add the concept of a "prechemistry avenue" to the preorganization idea (where prechemistry advocates that additional structural changes occur after a conformational change but before the chemistry in DNA Polymerase β, and that these structural changes drive the system towards a reaction state that is then congruent with the chemical step), which while an interesting idea, is entirely unrelated to the concept of preorganization.

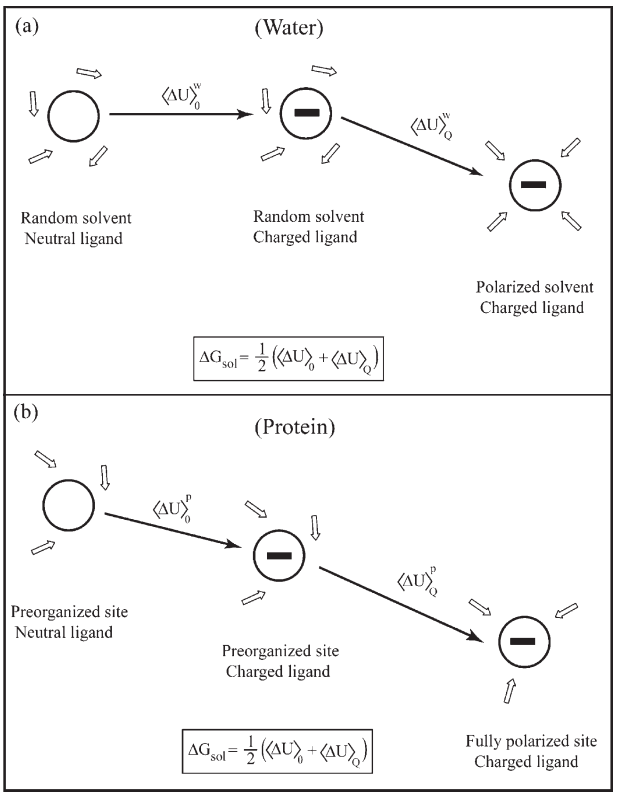

Figure 11

An illustration of the preorganization effect when considering (**A**) the stabilization of an ion in water by polarization effects, and (**B**) the stabilization of an ion in a protein by the cumulative effects of preorganization and polarization. This figure was originally presented in Ref. 203.

Similarly, the authors of Ref. 205 went even further, by not only renaming the preorganization concept to describe something fundamentally different, but even more dangerously, incorrectly using the terminology of the original preorganization concept in doing this.[205] That is, it was stated "*Warshel and coworkers have also stressed, via modeling, the importance of pre-organization for catalysis and KIEs. However, our perspective is that pre-organization is a transient, dynamical feature of the enzyme, whereas Warshel and co-workers conclude that sampling of multiple conformers does not, in fact, contribute significantly to the observed kinetic properties of enzymes*," despite the fact that this new definition of preorganization has nothing to do with the original definition of this term. Furthermore, the idea that the physics described by this new definition of "preorganization" can be related to catalysis is very problematic. That is, the sampling of multiple configurations before reaching the ES does *not*, in fact, contribute to catalysis at all, as was established in section "Free Energy Landscapes and Catalysis." Additionally, if the statement above is related to the time required to sample the TS region, then this time simply reflects the free energy barrier, but, of course, this is actually related to the reason the activation barrier changes, which is what catalysis is all about (see also section "Free Energy Landscapes and Catalysis," which discusses the relationship between free energy landscapes and catalysis). In other words, the sampling of multiple configurations simply *reflects* the free energy landscape, rather than *affects* it.

The difficulty with the preorganization concept is perhaps best illustrated with the case of ketosteroid isomerase (KSI), which is also one of the best examples of the preorganization effect. This case will be considered below.

The catalytic effect of KSI was originally quantified by the EVB calculations of Feierberg and Åqvist,[206] who reproduced the observed effect, and demonstrated its electrostatic origin. However, as argued earlier, this was not universally accepted, and Kraut *et al.*[147] chose the same system to attempt to demonstrate that the electrostatic effect cannot provide a major contribution to enzyme catalysis. In this work, the authors found that the binding energy of different substituted phenolate TSAs does not change significantly upon modification of the phenolates. What does change is the charge distribution of these phenolates, and thus the authors concluded that the electrostatic contribution to binding must be small in these systems, as well as with the actual TS and enolate intermediate of the KSI reaction. This conclusion was then assumed to represent valid experimental proof that electrostatic effects do not contribute to the catalytic power of KSI (and presumably that of other enzymes) in a major way. However, the authors reached this conclusion without any form of unique energy based analyses, and in fact completely contradict Feierberg and Åqvist's[206] careful study, which found that the catalytic effect of KSI is almost entirely due to electrostatic reorganization effects.

To resolve this controversy, we recently decided[203] to reanalyze the relationship between TSA binding and the chemical catalysis by KSI, as well as the binding of the transition state by means of theoretical simulations that do not make nonscientific speculations, but rather reproduce the relevant experimental results, and can thus be used to actually quantify the different contributions to the observed experimental effects. This study quantitatively reproduced the finding of a small electrostatic contribution to the binding of the TSAs, but also found a very large electrostatic contribution to the binding of the TS. The reason for this appeared to be the fact that the contributions from electrostatic preorganization to the binding of the enolate intermediate (and the TS) of KSI are significantly larger than the same effect for the binding of the TSAs. It was found that the difference between the TSA and TS is associated with the preorganization contribution ($\langle \Delta U \rangle_0$), which reflects the *nonpolar* state of the TSAs and the TS. The difference is that

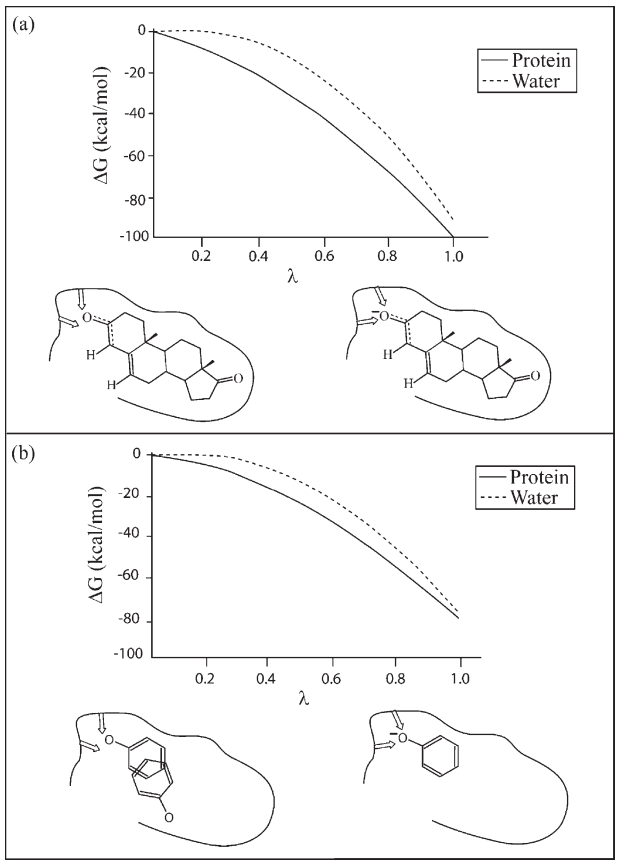

Figure 12

A comparison of the energetics of charging (**A**) the enolate intermediate in the active site of KSI and in water, and (**B**) the phenolate ligand in the active site of KSI. The preorganization effect is illustrated in the bottom part of each figure, and it can be seen that this effect is significantly smaller in the case of the phenolates, as once the ligand it converted to its nonpolar form, it is no longer held in a fixed orientation. This figure was originally presented in Ref. 203.

the TSAs are small and can rotate freely in the active site when they are in the nonpolar form and thus have a near zero preorganization contribution. On the other hand the TS cannot rotate, and this results in a large electrostatic preorganization term (see Ref. 203). These solid quantitative results significantly contradict the speculation of Kraut *et al.*,[147] who simply postulated that the electrostatic effect must be correlated with the presumed localization of the charge in the isolated phenolate, without quantitatively evaluating the corresponding electrostatic contribution (and thus without providing any way to quantify, assess or formulate either the electrostatic or in fact any other contributions). We realize that the concept of preorganization is complex, but in view of its crucial role as by far the most important catalytic factor it is essential to be able to evaluate it, and to understand its nature.

More recent attempts of Herschlag and coworkers to overlook the analysis of Ref. 203 have led to a series of exotic proposals that could neither reproduce nor rationalize any catalytic effect, and also confuse the preorganization idea with the removal of water molecules. One of these works[207] provided an interesting challenge by pointing out that the reaction of a small ring system is accelerated by KSI. However, this interesting result was found to be completely consistent with the preorganization idea, since the TS of the small ring cannot rotate freely, even in the uncharged form.[208]

Another related issue is the attempt of Devi-Kasavan and Gao[66] to classify electrostatic stabilization of the transition state (which has been clearly formulated and defined from as early as 1978[202] as being a generalized solvation effect) as a desolvation effect is worrisome, particularly as there is no attempt to compare the electrostatic stabilization due to the environment in the protein and in water, and thus to actually examine the desolvation idea. In our opinion, the attempt to invert the meaning of the desolvation idea after it has already been shown by others to be incorrect is not useful.

Throughout this section, we have again emphasized two key points. One is the fact that despite attempts to reduce the discussion of dynamical effects to a "semantic" issue, semantics do in fact play a key role, as without clear-cut definitions, it is impossible to conduct scientific discourse. The concept of preorganization is a key example of this, as, as we have illustrated earlier, there are to the best of our knowledge now four completely different definitions (including our own) of our original concept. The result of re-defining terms in such an *ad hoc* fashion has been significant misunderstandings in the field, that have resulted in exhaustive searches for explanations that have not produced any quantitative concepts, and lead us no closer to understanding enzyme catalysis than before these explanations.

EXPERIMENTAL INFORMATION FROM NMR AND OTHER APPROACHES IS CLEARLY CRUCIAL TO OUR UNDERSTANDING OF PROTEIN ACTION

Our discussion up to this point may have left the reader with the impression that we are downplaying the value of information from NMR or other experimental studies for understanding enzyme action (or biology in general), but we would like to emphasize that nothing is further from the truth. NMR studies provide major information about, for instance, protein flexibility, or pK_a values, which can be directly relevant to studies of enzyme catalysis. What we do object to is the loose usage of NMR studies to define thermodynamic quantities as being dynamical effects, which we will elaborate upon below.

We like to start by pointing out that NMR studies of short timescale (i.e., sub-ns) side and main chain dynamics in proteins that have been very helpful towards understanding protein action in general. Because of the breadth of the field, here, we will only be highlighting a few key studies, and for more detailed analysis, we refer the reader to the reviews of Refs. 209 and 210.

As an example of the usefulness of NMR in understanding protein action, Akke and coworkers[211] have used side-chain methyl groups as local reporters for conformational transitions that take place in the microsecond regime. In this work, they examined human FK-506 binding protein (FKBP12), an enzyme that uses a common surface for bonding the substrate and in its dual role as an immunophilin and folding assistant. They found conformational dynamics on a timescale of ~130 μs for methyl groups located in the substrate binding pocket, demonstrating the plasticity of this pocket in the absence of the substrate. From this, they suggested that substrate recognition involves the rapid relative movement of a subdomain in the enzyme comprising residues Ala81–Thr96, and that these observed dynamics play an important role in facilitating the interaction of this protein with its many partners. Although the actual role of dynamics in the biding process is still unclear, we have here a challenging and thought provoking issue.

NMR relaxation studies have also been useful in probing the long-range effects of substrate binding to an enzyme. An example of this is Oljeniczak and coworkers' study[212] of the changes in the backbone dynamics of the phosphotyrosine-binding domain (PTB) of the insulin receptor substrate 1 (IRS-1) when complexed with a Tyr-phosphorylated peptide derived from the interleukin 4 (IL-4) receptor. This work used NMR relaxation techniques to examine the changes in the dynamics of the backbone nitrogens of the IRS-1 PTB domain in both the free protein and the complex between the protein and the IL-4 receptor phosphopeptide, demonstrating that the backbone nitrogens of several key residues which make important contacts to the ligand are motionally restricted in both the free and complexed protein, with other residues only becoming motionally restricted *after* ligand binding (this included several residues that do not have any direct contact with the ligand). Additionally, increases in order parameters and internal correlation times were observed in residues in the loop between the β3 and β4 strands, which are located on opposite sides of the peptide-binding site. Taken together, this suggests that substrate binding can result in long-range reductions in mobility as a result of indirect contacts with the IL-4 peptide, mediated by the adjacent loops and β-strands.

NMR studies have been utilized to try to evaluate the role of changes in conformational entropy in elucidating protein function. For instance, Lee and coworkers[213] have performed an in-depth study of the response of the internal dynamics of calcium-saturated calmodulin to the formation of a complex with a peptide model of the calmodulin-binding domain of the smooth muscle myosin light chain kinase. Here, it was found that upon complex formation, the calmodulin backbone is unaffected by the binding of the domain, whereas the side chain dynamics are significantly perturbed. This was interpreted in terms of a heterogenous enthalpy/entropy partition. Now such studies can indeed help in decomposing entropy contributions into those of the substrate and those of the solvent (or protein), and, in fact, this issue has been the subject of several recent studies of enzyme catalysis (e.g., Refs. 153, 154, 214) as well as that of works which attempted to decompose the entropic contribution into its different components.[215] In such cases, comparison to NMR findings would be of enormous importance.

Another key use of NMR information is, in fact, the detection of slow conformational motions (as was done in e.g., Refs. 15, 28, 29, 34, 70, 73–75) which is being explored in the present work. Such information is useful in that it presents a well-defined set of facts, which should be reproduced by careful simulation studies. However, as clarified in the rest of the review, we believe that there are major problems with the interpretation of these findings as being dynamical effects that aid in the chemical step. Finally, even with regards to the pure dynamical proposal, we believe that NMR studies can aid in shedding major light on the question, by for instance the use of two distinct probes (one for the conformational and one for the chemical coordinates).

CONCLUSIONS

In recent years, the idea that enzyme dynamics can somehow contribute to catalysis has rapidly gained popularity, and been the subject of significant experimental and theoretical investigation. In this review, we have examined the most compelling arguments both for and against this proposal, and we conclude that existing studies have still not provided any unique evidence that enzyme dynamics makes a significant contribution to the catalysis of ground-state reactions. That is, although enzymes have evolved to dramatically lower the activation free energies of chemical reactions, no enzyme has actually been shown to increase the transmission factor by more than a factor of ~3 compared to that for the same reaction in water.

We should note that we have already published previous works[20,25,61] on this topic, and in our current review, we take into account the vast body of experimental and theoretical work that has been published since then. We remind the reader that a recent high profile review[27] has considered our previous work[61] a source of "confusion" in the field, as well as misleadingly crediting other workers (e.g., Refs. 216 and 217) with our findings

that dynamical effects have *no* contribution to the overall rate. The assertion of Ref. 27 is extremely problematic: our previous work[61] was a clear attempt to try to clarify the misconceptions in the field, and to move behind the idea that everything could be defined and redefined as a gray area. Unfortunately, the idea that protein motions have a role in catalysis has been strongly supported by some of the most prolific workers in the field for a long time (for examples, see for instance Refs. 29, 31, 125, 139 amongst others). This includes the clear suggestions[77] that both protein structure and dynamics have coevolved synergistically and that there exists a "*pre-sampling of conformational substrates before catalysis that are harvested for catalytic turnover.*" Other similar statements are presented in the "Introduction" section. Thus, the attempts of the some supporters of the dynamical proposal to now claim that dynamics does *not* actually contribute to catalysis[27] is one of the major sources of misunderstanding in the field.

The other source of misunderstanding is due to the fact that many of the versions of the dynamical proposal do not clearly formulate what the authors actually mean by it, making it hard to know what to look for in attempts to verify these claims. Further misunderstandings are associated with the tendency to use soft (changing) definitions and even to adopt key concepts such as the preorganization idea and to completely redefine these concepts. Thus, a large part of this review was about trying to clarify what the actual issues are to the reader, and to provide clear scientific definitions that let one judge the validity of the dynamical proposal for oneself. This problematic aspect of the field has been discussed extensively in sections "Free Energy Landscapes and Catalysis," "Tunneling and Other Nuclear Quantum Mechanical Effects," and "Enzyme Catalysis Is Due to Polar Preorganization, But This Has Little to Do With Recent Dynamical Definitions of Preorganization and Reorganization" where we try to clarify to the readers that only clearly defined proposals can be used when analyzing ideas about enzyme catalysis and that if the proposal is not defined in terms of some reaction coordinate and energy surface it cannot be explored, validated or discussed in logical terms. The complexity of the field and the difficulty with understanding the polar preorganization idea might be a part of the problem. However, recent attempts[95,139–142,149] to recast the complexity of the features of the free energy landscape as the solution to this question are problematic in themselves. To clarify this issue, we invested significant effort in sections "Free Energy Landscapes and Catalysis," "Tunneling and Other Nuclear Quantum Mechanical Effects," and "What About Conflicting Theoretical Findings?" on showing that concepts like "landscape searches," "entropy funnels" and "promoting motions" cannot account for any catalytic rate enhancement. We also emphasized that the best way to follow our point or to understand the problems with the other proposals it to try to formulate these proposals in a physically meaningful way (e.g., Figs. 5, 7 and 10). Otherwise, one ends up with a completely circular discussion and no way to reach any conclusions.

In order to highlight the problems that arise when the dynamical proposal is not formulated in a physically meaningful way, we would like to draw the attention of the reader to the definition used in Ref. 205, where "motions that contribute to catalysis" are defined as "*any motion that, if impeded, would reduce the ability of the enzyme to function by the mechanism that it has evolved to execute*". Here, conformational changes, allosteric transitions and protein reorganization are all brought up as examples of such important motions. We start by clarifying that the fact that even when protein conformational changes are essential to the function of proteins (for example by allowing binding) it does not *necessarily* follow that they are relevant to catalysis (or, in other words, that they are either causing or contributing to the rate enhancement). Now the main logical problem in the definition above is the implication that identifying a factor that's absence will "kill" the enzyme will tell us anything about its possible contribution to catalysis. The most glaring example of this is the requirement for protein folding. That is, even though the folding of the protein is *absolutely crucial* for catalysis, as it creates the reorganized active site environment, the folding timescale (or, if one so wishes, the rate of the release from the ribosome or the transcription process) has nothing to do with catalysis (i.e. no relationship exists). Of course, if a protein motion is important for the function of an enzyme, then impeding it would effectively "kill" the enzyme and thus have the ultimate effect of precluding catalysis, but assuming thereby that this motion helps drive catalysis in and of itself is flawed logic.

Additionally, it should be noted that the definition used here by Ref. 205 is not the definition that is used by the wider dynamics community, where works such as Refs. 27 through 31 effectively argue that the protein dynamics somehow facilitates catalysis by transferring energy to the chemical coordinate. Using a non-standard definition of "motions that contribute to catalysis" than that used by the dynamics community at large, without clarifying that this is different (and in the process also mixing function with catalysis) may lead to misunderstandings about the dynamical proposal.

Overall, we believe that the above definition of dynamical contributions to catalysis is symptomatic of a wider problem in the field, which involves the systematic confusion of chemical catalysis with the function of the enzyme. This confusion is highlighted by the considerations of Ref. 78 (see also below), which states "*Both the interaction of the Arg 55 side chain with the substrate, which facilitates the chemistry, and side-chain motions*

throughout the dynamic network, which enable facile interconversion of conformational states, are necessary for catalysis. Therefore, neither the dynamics nor active-site chemistry that lowers the transition-state energy are sufficient to promote efficient turnover" thus overlooking the key issue which is the requirement of having a clear concept about what is a chemical catalysis (and in the process providing two sequential and contradicting statements in one paragraph). In any case, in the interest of thoroughness, we have examined the vast wealth of experimental and theoretical work that has argued both for and against dynamical contributions to catalysis. After covering most probably all reasonable definitions of dynamics and catalysis, we concluded that there is currently no single experimental study that proves that dynamical effects contribute to chemical catalysis, and that all consistent theoretical studies conclude that there are in fact *no* significant dynamical contributions to enzyme catalysis.

Now despite the very slow realization of the problems with the dynamical proposals, and the gradual change in the formulation of this proposal, different incarnations of this idea persist in appearing in the most prominent journals, and being presented as the most exacting directions in the study of enzyme catalysis. One of the best examples of this is a very recent work,[78] where a detailed exquisite X-Ray and NMR study of Cyclophilin A found that a Ser99Thr mutation is capable of reducing k_{cat} by a factor of ~70, while slowing down the rate of interconversion between the two configurations by a similar factor (and also changing the equilibrium between the two conformations). The authors use this observation to argue that the parallel changes in the interconversion rate and catalysis indicate that the dynamics of the interconversion must therefore be crucial to catalysis (a highly questionable logical conjecture, which is likely to lead to as many misunderstandings as its predecessor, i.e. the argument that since the conformational change (lid closure) and the catalytic process are on similar timescales, therefore the conformational change must be related to the chemical catalysis).[29] Now although observing the relevant conformational changes is important as it finally offers the opportunity for consistent studies of the type we reported in Section "Consistent theoretical studies have established that there is no dynamical coupling between the chemical and conformational coordinates," this observation is likely to be a reflection of a rather trivial effect, that has little to do with any dynamical factor. That is, all that was actually found in Ref. 78 is that the Ser99Thr mutation reduces the rate by pushing Arg55 to a position that reduces its catalytic effect (and, in the process, destroys the corresponding preorganization). This is a simple and well understood allosteric effect (see e.g. our analysis of the Ras/GAP system[218]), which has nothing to do with the authors' idea that residue 99 is involved in some sort of "traffic-jam," as the native enzyme already has Arg55 in the correct position, and thus no motion is needed in order to move this system to the correct place (see Fig. 7 and the corresponding discussion for a clarification of this issue). In fact, the term "fluctuating due to the mutation"[78] is highly problematic, as the mutation is the process of generating another protein and the corresponding structural change does not represent a fluctuation. Now even if what is meant is actually the coupled motion between the two structures, this also does not provide new insights into catalysis. Rather, what we have here is a simple case of two configurations, where the conformational barrier changes from about 16 to 18 kcal/mol, and the chemical barrier from 13 to 15 kcal/mol. The origin for this fact should (and can) be explored by means of free energy calculations, rather than by presuming that it reflects the hindrance of some motion in a traffic-jam. In fact, our computational work (discussed in Section "Consistent theoretical studies have established that there is no dynamical coupling between the chemical and conformational coordinates") has already established that it is close to impossible that the information in the motion about the conformational barrier (and presumed traffic-jam) is transferred to the motion over the chemical barrier (which is lower in the present case). Furthermore, if some kinetic analysis shows that the conformational barrier is rate limiting, then we will have a case which is trivially described by simple kinetics, and where, having trivial kinetics, this rate-limiting process would have little to do with dynamics.

Perhaps the *main* problem with the work of Ref. 78 (and related similar studies) is the fact that the entire analysis is devoid of basic physical considerations, and there is no attempt to formulate the observation in terms of a free energy surface and reasonable frictional simulations (which we have done routinely). As long as the collective coupled motion follows Boltzmann's law, it is not useful to invoke dynamical factors. This is particularly crucial in this case, since the implication of the title and main text of Ref. 78 about "hidden alternative structures of proline isomerase" being essential for catalysis is unfortunately perhaps the best way to move away from properly understanding enzyme catalysis. That is, the "alternative" structures are *not* essential to catalysis, and implying that they are of crucial importance actually prevents advances in enzyme design, as it draws attention away from the real catalytic factors. More specifically, as catalysis occurs from the landscape of configurations with low activation barriers (see the analysis in Ref. 100), having several such configurations is not essential at all, and, following from this, alternating between high barrier configurations (as is the case in Ref. 78) is not even useful for catalysis.

In any case, the search for dynamical effects will undoubtedly continue, generating additional intriguing results and providing an active meeting ground for investigators with new experimental and computational

approaches. Our thesis is simply that in order to demonstrate a dynamical effect, one must show that it contributes significantly to catalysis in the enzyme and does not occur in the same reaction in solution, and we have yet to find a work that fulfills this simple requirement. Here it may be useful comment on the recent tendency to argue that we use a "limited definition of catalysis." First of all, catalysis has no meaning without a reference state, and second of all, any vague implication of dynamical rate enhancement must show what such enhancement is relative *to*, and even if it is defined as the activity of the native enzyme relative to its mutants, we must show that this is actually due to dynamical effects. To date, no such demonstration has been provided.

In summary, we like to emphasize that, as discussed in this work, there is no reported experiment that actually established any dynamical coupling between the conformational and chemical coordinates. On the other hand, the dynamical proposal has been finally explored by a theoretical study that actually simulates the coupling between the conformational and chemical motions and finds that the conformational motions do not affect the chemical rate constant. Of course, exploring this conclusion in detail is still a challenge that remains for future experimental and theoretical studies. But, what is currently the only direct consistent analysis in the field concluded that the chemical step does not remember the conformational motion, and thus that dynamics does not play an important role in catalysis and is unlikely to be the future of enzymology (and, in particular, the understanding of enzyme catalysis) for the 21st century.

TECHNICAL BACKGROUND

Computational approaches such as standard molecular mechanics simulations do not include electronic quantum effects, and therefore have to be combined with other approaches in order to be applicable to enzymatic reactions that exhibit quantum effects. Such hybrid quantum mechanics/molecular mechanics (QM/MM) methods[41] can in principle take into account the entire enzyme, but most are limited by their general neglect of entropic effects (though some specialized approaches however did attempt to address entropic effects as well[25]). Thus, the use of hybrid QM/MM techniques and a classical molecular dynamics approach allow for a wide-ranging analysis of the system at hand. Although classical MD simulations do not take into account zero-point energies and tunneling, these effects can be incorporated by a number of different methods, some of which are illustrated below. MD techniques such as umbrella sampling allow for the generation of free energy surfaces and the associated energetic barriers even for rare events, thus, combining such techniques with quantum mechanical considerations may provide a breadth of information about the system.

Now, to be able to gain a quantitative understanding of enzymatic reactions (as well as the corresponding reference reactions in solution, it is essential to be able to accurately calculate free energy profiles for these reactions, and, in recent years, the hybrid QM/MM approach has become the key tool for calculating protein function in general, and for studying chemical processes in proteins in particular (see for instance Refs. 219–233 to name but a few examples). However, despite the advances described earlier, we have not yet reached a stage where it is possible to use QM/MM approaches in fully quantitative studies of enzyme catalysis, as a quantitative evaluation of the potential surfaces for the reacting fragment should involve *ab initio* electronic structure calculations, which are too expensive to allow for the extensive configurational averaging that is necessary for proper free energy calculations (as using a QM/MM approach without proper sampling is not so effective[228]), despite the recent explosion of studies that use energy minimization approaches without proper sampling (for instance those of Refs. 150, 229, and 230 amongst others).

These problems can be overcome to some extent by specialized approaches that allow us to move towards *ab initio* QM/MM free energy calculations (such as those introduced in Refs. 231–233), but even these approaches are still in a development stage. Thus, we believe at present, the most reliable tool for understanding protein function is the EVB approach, which calibrates the system to the energetics of the reference solution reaction. That is, the EVB is a proper QM/MM method, that describes chemical reactivity by mixing resonance (or more precisely diabatic) states that correspond to the classical valence-bond (VB) structures describing the reactant, intermediate(s) and product states, the potential energies of which are represented by classical MM forcefields of the form:

$$\varepsilon_i = \alpha^i_{\text{gas}} + U^i_{\text{intra}}(\mathbf{R},\mathbf{Q}) + U^i_{\text{Ss}}(\mathbf{R},\mathbf{Q},\mathbf{r},\mathbf{q}) + U_{\text{ss}}(\mathbf{r},\mathbf{q}) \tag{12}$$

Here, **R** and **Q** represent the atomic coordinates and charges of the diabatic states, and **r** and **q** are those of the surrounding protein and solvent, α^i_{gas} is the gas-phase energy of the *i*th diabatic state (where all the fragments are taken to be at infinity), $U_{\text{intra}}(\mathbf{R}, \mathbf{Q})$ is the intramolecular potential of the solute system (relative to its minimum); $U_{\text{Ss}}(\mathbf{R}, \mathbf{Q}, \mathbf{r}, \mathbf{q})$ represents the interaction between the solute (S) atoms and the surrounding (s) solvent and protein atoms, $U_{\text{ss}}(\mathbf{r}, \mathbf{q})$ represents the potential energy of the protein/solvent system ("ss" designates surrounding–surrounding). The ε_i of Eq. (12) forms the diagonal elements of the EVB Hamilto-

nian (H_{ii}). The off-diagonal elements of the Hamiltonian, H_{ij}, are represented by simple exponential functions of the distances between the reacting atoms. The H_{ij} elements are assumed to be the same in the gas phase, in solutions and in proteins, and the ground state energy, E_g, is then obtained by diagonalizing the EVB Hamiltonian.

The relevant activation free energies ($\Delta g^{\ddagger}$) are then obtained by adiabatically changing the system from one diabatic state to another. In the simplest case, which only involves two diabatic states, this "mapping" potential ε_m) can be written as a linear combination of the potentials of the reactant and product, i.e., ε_1 and ε_2 respectively:

$$\varepsilon_m = (1 - \lambda_m)\varepsilon_1 + \lambda_m\varepsilon_2 \qquad (0 \leq 1 \leq 1) \qquad (13)$$

where λ_m is changed from 0 to 1 in $n + 1$ fixed increments ($\lambda_m = 0/n, 1/n, 2/n, \ldots, n/n$), and potentials with one or more of the intermediate values of λ_m force the system to fluctuate near the TS.

Finally, the free energy, ΔG_m, associated with changing λ_m from 0 to m/n is evaluated by the free energy perturbation (FEP) procedure (which has been described in detail in e.g., Ref. 21 amongst others). However, after obtaining ΔG_m, we also need to obtain the free energy that corresponds to the adiabatic ground state surface along the reaction coordinate, x. This free energy (which is referred to as a "free energy functional") is obtained by the FEP-umbrella sampling (FEP/US) method.[21,64] The main point for purpose of doing this is that the FEP/US approach may be also used to obtain the free energy functional of the diabatic states by the equation below[21]:

$$\Delta g_l(x') = \Delta G_m - \beta^{-1} \ln \langle \delta(x - x') \times \exp[-\beta(\varepsilon_1(x) - \varepsilon_m(x))] \rangle_{\varepsilon_m} \qquad (14)$$

Here, e_m is the mapping potential that keeps x in the region of x'. If the changes in ε_m are sufficiently gradual, the free energy functional $\Delta g(x')$ that is obtained with several values of m overlap over a range of x', and the complete free energy curve for the reaction can be obtained by patching together the full set of $\Delta g(x')$, and the energy gap ($x = \varepsilon_1 - \varepsilon_2$) is generally defined by the generated reaction coordinate, x. This selection[21,234] is particularly powerful when one tries to represent the entire many dimensional solvent space by a single coordinate (see Ref. 64).

Now, in recent years, the effectiveness of the EVB (and/or the energy gap mapping) has been recognized by many workers other than us (e.g., Refs. 122, 182, 235 just to name a few works), and, perhaps the strongest indicator of its increasing popularity are recent attempts to repackage the EVB under a different name (see e.g., Refs. 236–238). Some of these studies are extremely, problematic as clarified in our recent discussion[239] on a work of Truhlar, Gao and coworkers.[240] In any case, the EVB is, at present, the most powerful approach for quantitative evaluations of activation free energies in enzymes and in solution. The quantitative value of this method has already been demonstrated in the challenging calculations of the effect of mutations on the catalytic reaction of dihydrofolate reductase (DHFR),[127] where distant mutations have a significant effect, and being able to do this accurately in fact requires averaging over several calculations, which has been recently accomplished by others.[241] Furthermore, the power of the EVB approach has also been demonstrated in benchmarks for enzyme design.[242] This issue is crucial, since if reliable free energy calculations are able to reproduce the observed rate constant and catalytic effect, then it is hard to argue that other effects are missing.

To get the full rate constant of Eq. (2), we also need to evaluate the transmission coefficient (κ). This factor at least formally[44] contains all dynamical effects (although we will consider many other definitions). Much of the discussion of whether or not dynamical effects contribute to enzyme catalysis has therefore revolved around whether or not κ is higher in enzymes than in solution. If we temporarily neglect tunneling and other quantum effects, κ depends on two interrelated factors: (a) the probability that a system arriving at $x^{\ddagger}$ from the reactant side of the barrier will end up on the product side rather than regenerating the reactants, and (b) the average number of times that a productive trajectory passes back and forth across $x^{\ddagger}$ before it permanently moves to the product side. These factors can be evaluated by examining a family of MD trajectories that start in the TS with a thermal distribution of velocities.[25,42–44,64] The trajectories are propagated both forwards and backwards in time until both segments have settled in either the reactant or the product state, and the forward and backward segments are combined to obtain a complete trajectory.

One way to calculate the transmission coefficient, called the "reactive flux" method described elsewhere.[243–245] The transmission factor also can be obtained by considering the average effective velocity with which productive trajectories cross the TS.[25,64] This can be converted to an expression related to the autocorrelation of the EVB energy gap,[25,64] which is in turn related to the autocorrelation that has been widely used in studies of solvation dynamics.[246–250] In fact, despite the elegance of the reactive flux method, the autocorrelation of the energy gap has the added advantage that it provides a more direct connection to the view of the enzyme as an effective solvent for the reacting groups. The transmission factor can also be evaluated by the EA-VTST[251] method.

Simulation studies of dynamical effect should also be able to explore nuclear quantum mechanical quantum

mechanical (NQM) effects. This can be done by using the quantum classical path (QCP) approach of Warshel and coworkers,[191,252] which is based on the centroid version[45,253,254] of Feynman's path integral approach,[255] but involves a major practical improvement, based on using a classical reference potential and classical trajectories to evaluate the quantized free energy (by using a perturbation treatment for the move from the classical to the quantized potential). That is, calculating the centroid probabilities in condensed-phase reactions is very challenging, as it involves major convergence problems, and the QCP approach therefore offers an extremely effective (and simple) way to evaluate this probability, without making any significant changes to the simulation program. This is done by taking the classical potential surface of the reacting system as a reference potential, propagating trajectories on this surface, and then using the position of the classical atoms to generate the centroid position for the quantum mechanical partition function.

It is worth noting that QCP studies[61,96] that have focused on comparing the NQM effects in enzyme and solution have found that the corresponding corrections are similar. It is also important to note that our QCP approach has been recently adopted by other workers, though sometimes under the guise of a different approach.[158] Also, Hammes-Schiffer *et al.* employed a molecular dynamics with quantum transitions (MDQT) surface hopping method,[256] in combination with the reactive flux approach[257] in their studies of dihydrofolate reductase (DHFR).[258,259] Finally, the ensemble-averaged variational transition state theory with multidimensional tunneling (EA-VTST) approach[250,260] has also been used to calculate the magnitude of quantum effects on nuclear motions.

ACKNOWLEDGMENTS

The authors thank Dr. Andrei Pisliakov and Dr. Spyridon Vicatos for their assistance in the preparation of the artwork for this manuscript.

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