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Perspective: Defining and quantifying the role of dynamics in enzyme catalysis

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Enzymes control chemical reactions that are key to life processes, and allow them to take place on the time scale needed for synchronization between the relevant reaction cycles. In addition to general interest in their biological roles, these proteins present a fundamental scientific puzzle, since the origin of their tremendous catalytic power is still unclear. While many different hypotheses have been put forward to rationalize this, one of the proposals that has become particularly popular in recent years is the idea that dynamical effects contribute to catalysis. Here, we present a critical review of the dynamical idea, considering all reasonable definitions of what does and does not qualify as a dynamical effect. We demonstrate that no dynamical effect (according to these definitions) has ever been experimentally shown to contribute to catalysis. Furthermore, the existence of non-negligible dynamical contributions to catalysis is not supported by consistent theoretical studies. Our review is aimed, in part, at readers with a background in chemical physics and biophysics, and illustrates that despite a substantial body of experimental effort, there has not yet been any study that consistently established a connection between an enzyme's conformational dynamics and a significant increase in the catalytic contribution of the chemical step. We also make the point that the dynamical proposal is not a semantic issue but a well-defined scientific hypothesis with well-defined conclusions. *Published by AIP Publishing.* [http://dx.doi.org/10.1063/1.4947037]

I. INTRODUCTION

Enzymes provide enormous rate acceleration to chemical reactions and thus are able to regulate and synchronize the key chemical reactions that make life possible. In addition to the interest in the biological role of enzyme, there is a major long-standing interest in elucidating the reason for the enormous catalytic proficiencies of enzyme. This interest has led to many proposals that tried to rationalize the source of enzyme catalysis (for a partial list, see Refs. 1 and 2). Although one of these proposals (i.e., electrostatic based catalysis[1,3,4]) accounts for the majority of the magnitude of the catalytic effects, the other proposals are still very popular. Arguably the most popular alternative proposal is the idea that the catalysis is due to dynamical effect. Here, there have been some semantic problems that made it hard to bridge between qualitative and quantitative definitions of what constitutes a catalytically important dynamical effect (see discussion in, e.g., Refs. 2 and 5). This review, therefore, will attempt to rigorously define and describe the dynamical proposal, and to define its possible contributions to enzyme catalysis. In doing so we will also clarify that it is not justified to argue that the difference between opposing proposals is not substantial and can be simply attributed to semantic misunderstandings.

Although the explosion in interest in dynamical contributions to enzyme catalysis is relatively recent, the proposal originated at least 35 years ago with the work by Careri, Gratton, Werber, Karplus, and others,[6–9] before attracting major attention in the past two decades (see, e.g., Refs. 10–22, among many others). Although serious concerns have been raised about this proposal,[23–29] it remains popular and articles in its defense continue to be published in leading journals.[20,30–42] In order to explore the dynamical proposal, we have to recognize that in contrast to some recent works, that argued that our attempts to address the dynamical proposal just leads to confusion in the field,[43] it is in fact quite simple to address this issue in a completely rigorous scientific way. To achieve this, it is imperative to avoid semantic confusion and use very clear definitions of what is meant by dynamical proposals. In this context, we point the readers to a recent review[41] that eloquently argued that the confusion about the dynamic proposal is due to the fact that earlier works did not clarify what they meant by dynamics (e.g., not defining inertial or diffusive models), and that Ref. 41 does instead focus on stochastic dynamical models (which it called passive dynamics), since this is what is implied by Marcus-like models. However, as we illustrated with explicit examples in our previous review,[2] it is very clear that the majority of these works explicitly refer to dynamical contributions to catalysis, and stochastic approaches are not dynamical and thus are not a part of a correctly formulated dynamical proposal. This problem extends to the puzzling implications[41] that enzymologists knew and meant that dynamics is related to statistical models, which is unsupported by the literature. Additionally, as far as Marcus-like models (which we introduced consistently to solution and enzymes in the correct adiabatic limit in 1990[44]

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and 1991[45]) are concerned, the attempts to use such models in analyzing tunneling, in, for instance, all the arguments and discussions in early and ongoing works,[12,15,16,46] seem to clearly be attempts to promote the problematic dynamical proposal. This does not involve an attempt to support or explore the relationship between the activation free energy and the electrostatic preorganization.[1,3]

Fortunately, the key proposal about the catalytic power of enzymes has already been proposed and formulated. Thus, what was meant by different proposals can be clearly established, without being sidetracked by the names that either have been or are now being used to describe such proposals. Of course, to eliminate confusion, it is crucial to have clear logical and scientific definitions of what is meant by the dynamical proposal. Thus we will examine and analyze this proposal within the framework of clear, physically based definitions of dynamical contributions to catalysis, in order to demonstrate that within rigorous frameworks, there is no need to invoke dynamical contributions to rationalize the observed catalytic effects. We also like to clarify (as will be discussed below) that conformational sampling gives entropic contributions, but these again have nothing to do with dynamics but rather with the available configurational space. Thus, after presenting our arguments, we will leave it to the readers to decide whether, within rigorously defined frameworks, it is still possible to argue for dynamical contributions to enzyme catalysis.

II. FUNDAMENTAL DEFINITIONS

The starting point of this review is the definition of enzyme catalysis by Figure 1, where we compare the reaction in the enzyme to a reference reaction in water. Now, as established in Ref. 4, the most relevant reference state is the "chemically filtered"[4] reference reaction, which is essentially the enzymatic reaction without the enzyme. In this way, it is not necessary to consider the trivial issue of the energetics of changing the mechanism as part of the catalytic puzzle, since the change in mechanism in water can be taken into account separately. We also find it advantageous to consider the reference solution reaction as occurring when all the reactants are in the same solvent cage, where k_{cage} is approximately 55 times larger than the rate constant in standard conditions (for rigorous definition see Ref. 47).

In determining the magnitude of the dynamical contribution we should explore the rate constant (k_{cat}) that corresponds to the activation barrier of the chemical step using

$$k = \kappa k_{TST}, \tag{1}$$

where k_{TST} is the rate constant obtained by transition-state theory (TST),

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

and κ is the "transmission coefficient," while x is the generalized reaction coordinate (for more details see Ref. 1). Note that in our formulation, the activation barrier can include quantum corrections (which are included in the pre-exponential factor in other treatments). At any rate, our task is

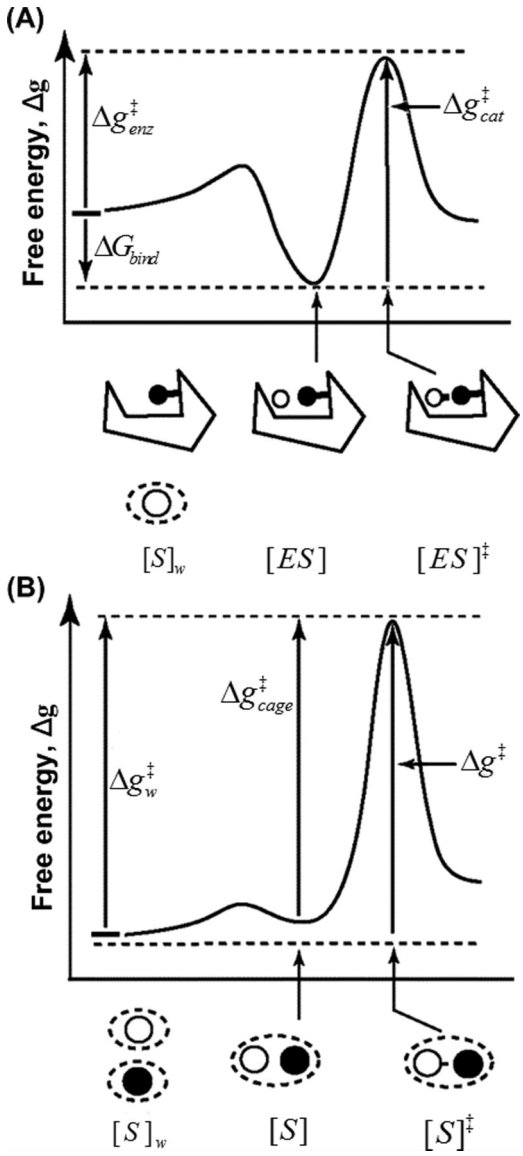

FIG. 1. Schematic illustration of the free energy profiles for (a) a model enzyme-catalyzed reaction and (b) the corresponding reaction in aqueous solution. ΔG bind denotes the binding free energy of the substrate, $\Delta g^{\ddagger}_{enz}$ and $\Delta g^{\ddagger}_{w}$ denote the activation barriers for the reactions in enzyme and in solution respectively, $\Delta g^{\ddagger}_{cat}$ and $\Delta g^{\ddagger}_{cage}$ describe the corresponding energetics from the Michaelis complex and a solvent cage. The activation free energies $\Delta g^{\ddagger}_{enz}$ and $\Delta g^{\ddagger}_{cat}$ are associated with k_{cat}/K_M and k_{cat}, respectively. Reprinted with permission from Warshel *et al.*, "Electrostatic basis for enzyme catalysis," Chem. Rev. **106**, 3210 (2006). Copyright 2006 American Chemical Society.

to determine what are the contributions that make k_{cat} larger than k_{cage}.

In examining the importance of dynamical effects, one must start with clear definitions, trying to avoid the impression that our findings depend on the definitions used. In doing so, we disagree strongly with recent proposals that the confusion in the field is due to the omission of stochastic dynamics and landscape sampling from the considerations of the dynamical proposal (see, e.g., Ref. 41 and the Introduction). Of course, we note that the atoms are moving all the time but this fact cannot be used in supporting the dynamical hypothesis, as long as the chance for moving over barriers is determined by the corresponding Boltzmann probability.

Here we note that formally it has been shown that all the non-Boltzmann dynamical effects are expressed by the transmission factor,[48–50] where it was found that this factor does not contribute to enzyme catalysis.[1,2] However, we will consider here also other definitions of dynamical effects, including the reasonable definition of coherent non-Boltzmann motions, as well as the contributions of nuclear tunneling and the presumed effect of coupled modes.

In our view, it is almost impossible (except in the simplest of cases) to determine the importance and magnitude of dynamical contributions to chemical reactivity (and, clearly, to enzyme catalysis) without the use of computer simulations. *Such approaches have been gradually developed following our quantum mechanics/molecular mechanics* (*QM/MM*) *work in* 1976[51] *and our early study of the dynamics of enzymatic reaction* (e.g., see Ref. 26) and will thus only be mentioned here briefly. We note, however, that some of these approaches are sufficiently reliable to determine the observed activation free energies and the magnitude of mutational effects. More technical information about which will be given in Section X.

III. ENZYME CATALYSIS AND THE TRANSMISSION COEFFICIENT

One of the most obvious and formally rigorous ways to evaluate and examine the catalytic contributions of dynamical effects is to determine the transmission factor in the enzyme and in solution. The transmission factor can be determined in several ways. The most straightforward way is to examine the recrossing of productive trajectories once they arrived at the transition state (TS).[50] Another way is to use the "reactive flux" method.[52–54] An additional way is offered by evaluating the average velocity by which productive trajectories pass the TS. This average velocity can be obtained from the autocorrelation of the EVB energy gap.[27,45] A comparison of such autocorrelation for the reaction in the enzyme and the corresponding reaction in solution is given in Figure 2. As found in our studies,[2] it can be seen that the autocorrelation function and the transmission factor behave in a very similar way and therefore cannot provide dynamical contribution to catalysis. Furthermore, all actual evaluations of the transmission factors of both enzymatic and solution reactions (see discussion in Refs. 2 and 41) provide a value which is not much smaller than 1, and thus cannot make a significant contribution to any deviations from TST.

Works that seem to emphasise the importance of changes in the transmission factor can send a problematic message to those who support the dynamical catalytic proposal. As an example, we can then consider the interesting work of Ref. 55. This work examined the dynamics of the hydride transfer step of the enzyme *Escherichia coli* dihydrofolate reductase (EcDHFR) and has attributed the observation of non-linear Arrhenius plots to tunneling and recrossing effects, while not considering the possibility that the deviations reflect entropic effects (ignoring entropic effects could not account for the cases of large deviations, such as those in Section VIII C). However, the point of most concern is the chance that the finding[55] of transmission factors of 0.57 and 0.49 for the light and heavy variants of *Ec*DHFR, respectively, might enter into arguments about the importance of dynamical contributions to catalysis. Here we can note that there is a very small difference in the rate constant due to the transmission factor, but even without comparing to the same reaction is water, we can note that the dynamic contribution has very small effect when compared to the very large overall (more than 5 orders of magnitude[56]) catalytic effect.

IV. ENZYME CATALYSIS AND COHERENT FLUCTUATIONS

A. Defining the concept

Some readers may consider it unjustified focusing only on the transmission factor when examining dynamical

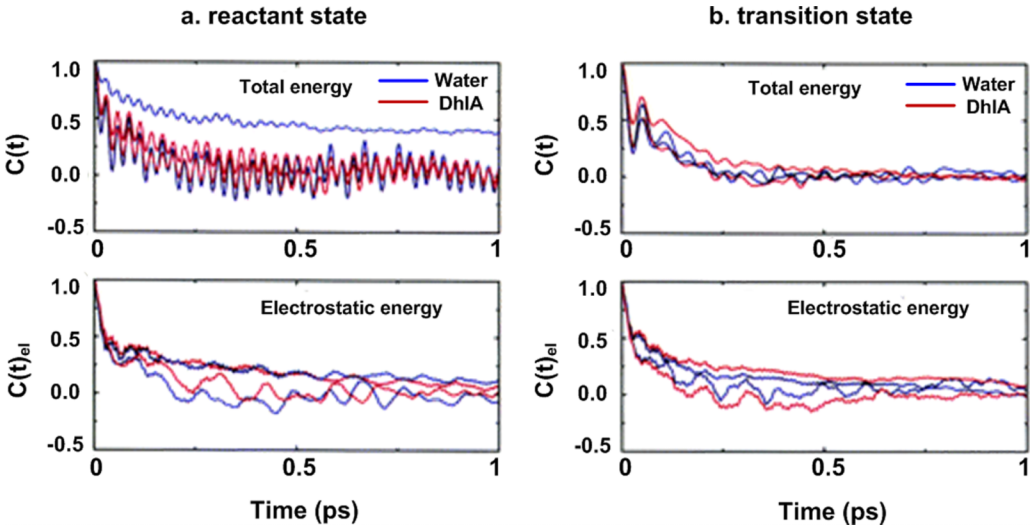

FIG. 2. Autocorrelation function of the energy gap between the reactant and product states in the region of the TS in halo alkane dehalogenase (red), as well as the reference reaction in water (blue). The plot on the top shows the total energy, whereas that on the bottom shows only the electrostatic contribution to the energy. The autocorrelation functions are normalized to 1 at zero time. Reprinted with permission from Olsson, M. H. M. and Warshel, A., "Solute solvent dynamics and energetics in enzyme catalysis: The S_N2 reaction of dehalogenase as a general benchmark," J. Am. Chem. Soc. **126**, 15167–15179 (2004). Copyright 2004 American Chemical Society.

contributions to catalysis, and therefore we consider here also other effects. For example, in cases where the first passage time involves non-Boltzmann coherent fluctuations, then there *is* a true dynamical effect. Such effects can occur in ultrafast light induced biological reactions (see, e.g., Ref. 57), but are unlikely to occur in thermally activated chemical reactions.

The idea that dynamical contributions to catalysis involve some type of coherent motion with a memory of the initial trigger (e.g., the movement from an open to a closed structure) has been implicitly or explicitly proposed in many high profile works, several of which have been discussed in detail in Ref. 2. However, as such dynamical proposals are almost never presented in a clear logical way. Our perspective is that the non-Boltzmann dynamical idea has a unique meaning, regardless of the way it was expressed. For example, and as also discussed in Ref. 2, the proposals put forth by Refs. 20, 31, 35, 36, and 58 must imply non-Boltzmann process. In our view, Figure 3 provides a clear definition of cases with dynamical contribution to catalysis, where, in an inertial model, the kinetic energy from processes such as the binding event is used to help pass the chemical barrier.

Propagating trajectories from the TS can also be used to explore short time scale dynamical effects. Such studies (e.g., Ref. 59) have indicated that the trajectories in both enzymes and solutions move randomly in the reactant state and correspond to the incoherent dynamics limit. Exploring this issue more consistently requires one to run very long time trajectories from the ground state to the TS or to separate experimentally the motion in the conformational and the chemical directions. Below, we will show that experimental studies have not yet managed to resolve this issue, but specialized theoretical works have provided compelling evidence against the inertial model.

B. Experimental studies with direct theoretical analyses

We will start this section with a brief discussion of the experimental NMR studies that generated most of the recent resurgence of interest in the dynamical proposal (e.g., Refs. 20 and 60–67). However, despite the overall great usefulness of NMR studies, there are problems with using such studies as a basis for defining the observed quantities as dynamical effects, and, more seriously, with attempts to define qualitative interpretations of experimental observations as experimental facts. This issue will be discussed below with just a few examples.

One of the main model systems used to advance the dynamical proposal has been the cyclophilin A (Cyp A) that catalyzes the isomerization of proline.[20,62,68,69] For example, it was found that the transverse relaxation of the catalytic Arg55 is accelerated in the bound substrate and reaches a rate that is

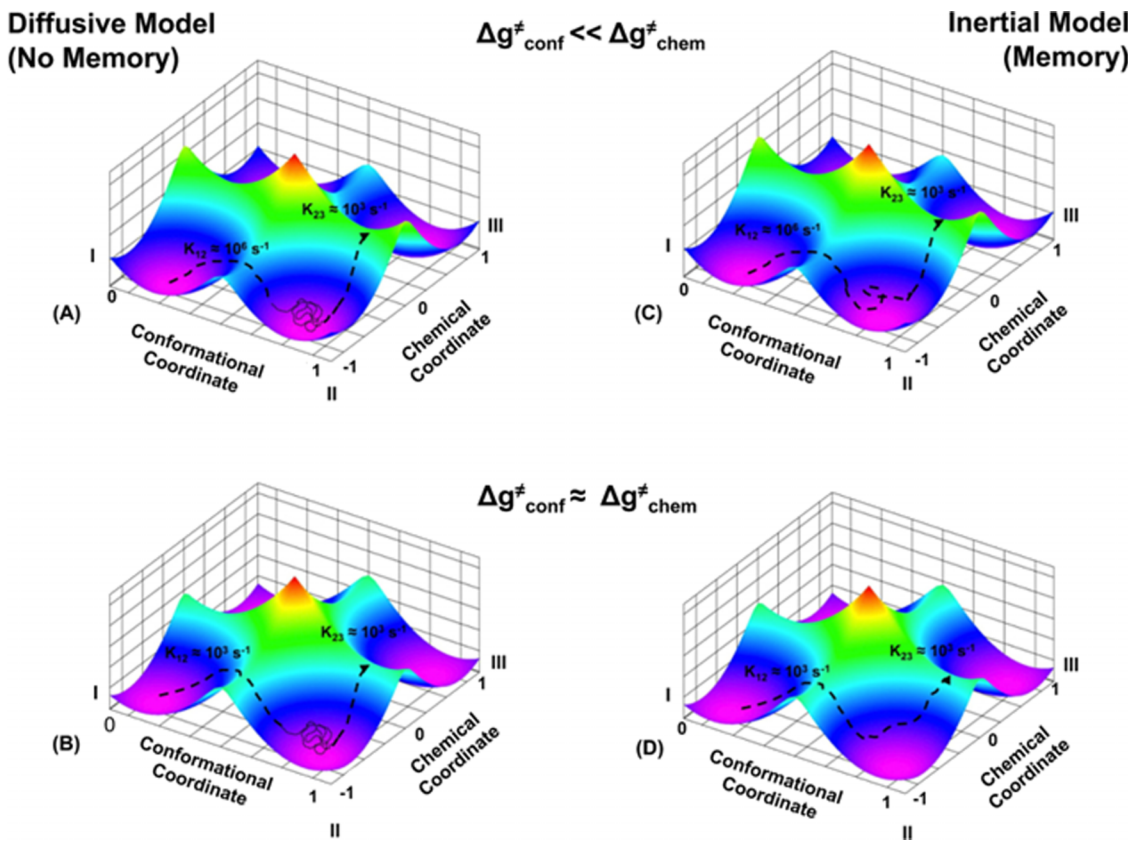

FIG. 3. A schematic depiction of the diffusive ((a) and (b)) and the inertial models ((c) and (d)). These two limiting models are shown in the case where the conformational barrier is much smaller than the chemical one (i.e., $\Delta g^{\neq}_{conf} \ll \Delta g^{\neq}_{chem}$, parts (a) and (c) at the 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). Reprinted with permission from Pisliakov, A. V., Cao, J., Kamerlin, S. C., and Warshel, A. "Enzyme millisecond conformational dynamics do not catalyze the chemical step," Proc. Natl. Acad. Sci. U. S. A. **106**, 17359–17364 (2009). Copyright 2009 National Academy of Sciences.

similar to the rate of the chemical step.[20] This was considered to be an indication of a dynamical coupling but as discussed in Ref. 2 we do not have a real observation of a coupling between the conformational and chemical steps.

A study of CypA tried to explore the role of the enzyme's conformational dynamics by replacing the change of the chemical barrier by an effective torsional barrier, and by running the simulations with different assumed barriers.[70] The authors concluded that the activation barrier controls the catalysis, and that the dynamical effects are actually anticatalytic by a minor factor of 1/10. Note, however, that this analysis might have some problems, since the considerations of the difference between the enzyme and solution have not correctly reflected the electrostatic differences, which would require a QM/MM description. Furthermore, most of the studies were confined to an unrealistic barrier range where diffusive effects are very important.

NMR studies[13,71,72] of the reaction of dihydrofolate reductase (DHFR) found that mutations of residues in a loop that undergoes relatively large backbone motions change the catalysis, and this was taken as a support to the dynamical idea. However, it is also very likely that the distant mutations simply change the preorganization and the TS stabilization.[56]

Another high profile work that has attempted to support the dynamical proposal and to challenge the catalytic preorganization idea has studied the effect of mutations that restrict the protein conformational changes.[40] In particular, this work argued that the mutational induced reduction in catalysis is due to dynamical effects, and also that the reorganization change upon mutation is very small and thus presumably not responsible for this change in catalysis (based on inspecting the observed structural changes upon mutation). However, our subsequent simulation study[73] has shown that the observed catalysis simply reflects the alternation of the free energy surface, and the corresponding changes in preorganization. It was also pointed out[73] that the preorganization cannot be estimated by just examining structural changes by experimental methods that cannot accurately determine the reorganization of hydrogen bonds, but rather, it is necessary to perform free energy calculations. Our clear analysis of these problems was subsequently confirmed by other groups,[55,74,75] although some obfuscated the fact that they have merely reproduced our previous results. In our overall consideration of the dynamical proposal, we would also like to draw attention to the findings of Miller and coworkers[76] who explored the difference between statistical and dynamical effects in DHFR. These authors concurred with our view, and found only a very small minimal role for (non-local) dynamical contributions.

Another key system that has been used extensively to support the dynamical proposal has been adenylate kinase[33,36,77] (which is phosphotransferase enzyme that catalyzes the interconversion of adenine nucleotides, and plays an important role in cellular energy homeostasis). For example, simulations and experimental studies led some workers to argue that there must be a link between the microsecond motions and the enzyme function.[31,58,78] Most notably, Ref. 35 has demonstrated that the ps to ns fluctuations of the enzyme are very similar in the mesophilic enzyme and a hyper thermophilic enzyme, at some temperatures. This led to the proposal of a "hierarchy of time scales," which were presumed to serve as a link between catalysis and function. This is, however, at best an indirect consideration, and, unfortunately (and despite major experimental and computational effort), we do not have any study that established a unique connection between the chemical step and the conformational dynamics.

Single molecule spectroscopy has provided insight into the dynamical properties of enzymes (see, for example, Ref. 79). Our analysis of the corresponding experiments is provided in Ref. 2. Overall, despite the elegance of the experiments discussed above, we conclude that, in cases of processes with high barriers, the only way to explore the inertial proposal is provide (at present) by using computer simulations, and such studies will be described in more detail below.

C. Consistent theoretical studies provide no support for inertial motion in enzyme catalysis

Considering the difficulties (and at present inability) to explore the inertial hypothesis by experimental studies, it seems that the only option is to develop a theoretical way of modeling the relevant long time dynamics along the conformational and chemical coordinate.

A major progress in this direction was provided by a simulation study[80] that used multiscale approach and provided a way for us to move to the millisecond (ms) time scale. This study exploited our renormalization approach[81] and studied the phosphoryl transfer reaction catalyzed by adenylate kinase as a model system, showing that the kinetic energy of the conformational motion is completely dissipated during the conformational motion and thus *cannot* affect the time scale for the barrier crossing during the chemical event (see Figure 4). Apparently the inertial coupling decays in less than nanoseconds and the barrier climbing is controlled by the Boltzmann probability of the productive trajectories. Our crucial finding is shown in Figure 4. It should also be emphasized that we reached the same conclusions moving from a 2-D model to a full CG protein-substrate model.[80]

D. Low barrier checkpoints and replication fidelity

A field where poorly defined "dynamical type effects" have been implicated to be involved is the control of replication fidelity by DNA polymerases (which is an enzyme that creates DNA molecules by assembling nucleotides, the building blocks of DNA. These enzymes are essential to DNA replication and usually work in pairs to create two identical DNA strands from a single original DNA molecule). The implication of dynamical effect in the polymerization reaction includes the idea that the difference in the selectivity of the right (R) and wrong (W) base pairs is due to pre-chemistry barriers that are, in an absolute scale, lower than the chemical barrier. Thus, this idea considered "checkpoints" as crucial factors in the control of the fidelity (see discussion in Refs. 82 and 83). Although we have clarified that this is a problematic idea, using well-defined free energy considerations,[2,82,83] it might be instructive to note a recent criticism[84] of our

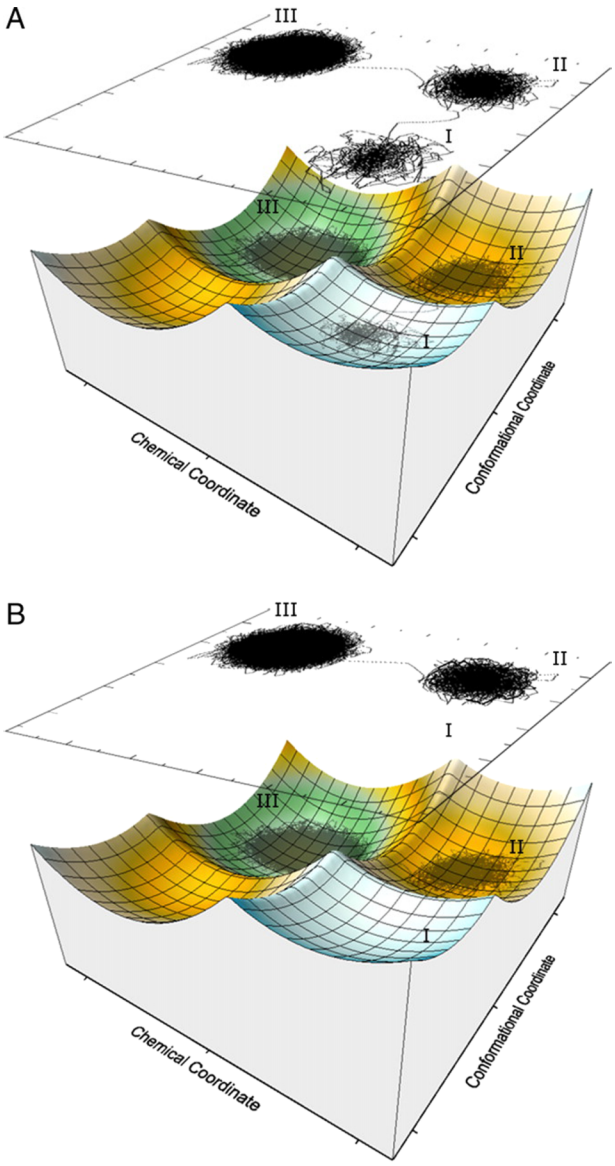

FIG. 4. Illustrating hypothetical effects of excess binding energy on the chemical step of enzyme catalysis. Two hypothetical scenarios are shown here: one where (a) the trajectory starts from a high-energy open state (I), rapidly moves towards the closed Michaelis complex (II) and finally crosses to the PS (III), and (b) the corresponding case where the simulations stats at the closed RS (II), and move to the PS (III) (b), based on simulations performed in Ref. 80. In both cases, the trajectory appears to be fully randomized in the closed reactant state, with no memory effect. Reprinted with permission from Pisliakov, A. V., Cao, J., Kamerlin, S. C., and Warshel, A., "Enzyme millisecond conformational dynamics do not catalyze the chemical step," Proc. Natl. Acad. Sci. U. S. A. **106**, 17359–17364 (2009). Copyright 2009 National Academy of Sciences.

arguments in Ref. 82 which, instead of actually addressing our very clear energy diagrams, has focused on the names used in some of our discussions. That is, while we did use (in the caption of Figure 1 of Ref. 82) the term "*rate*" when we meant "*specificity*," and "*rate determining*" when we again meant "*specificity*" (in the abstract), it should be very clear from all our figures and discussion that we meant that the fidelity is determined by the changes in the highest barrier, relative to the unbound state. Thus, arguments provided in Ref. 84 that, in contrast to our work, prechemistry *can* contribute to fidelity even if it is not rate-limiting, distorts what was shown in our actual paper (that there is no scenario where the prechemistry step contributes to the fidelity *if it does not present the highest barrier in the catalytic cycle*). Obviously, Figure 4 of Ref. 82 shows that the fidelity *can* be controlled by prechemistry steps, when these present the highest barriers.

V. MODE COUPLING DYNAMICAL EFFECTS AND COMPRESSION

It has been tempting to suggest that a special coupling between the protein vibrations and the chemical reaction coordinate contributes to catalysis[85–87] (note that such a proposal is, in some respects, similar to the inertial model discussed in Section IV). This has even been extended to the rather extreme proposal that our sense of olfaction is based on vibrational modes,[88] which is clearly really problematic (see, e.g., Ref. 89), and will therefore not be examined here.

The coupling between the protein vibrations and the chemical process can be explored by our dispersed polaron (DP) model.[27,45,59,90] This method evaluates the EVB energy gap during MD simulations and uses the corresponding autocorrelation function to determine the relevant power spectrum, $J(\omega)$, and then evaluates the contribution to the reorganization energy by

$$\lambda = \frac{1}{2}\sum_j \hbar\omega_j\delta_j^2 = \frac{\beta}{2\pi}\left|\int_{-\infty}^{\infty} J(\omega)\,d\omega\right|. \tag{3}$$

Using this framework, it is possible to return to studies of several different systems[91–93] that were used to argue for the mode coupling idea. In this respect we like to clarify that having correlated motions does not present a new view on catalysis because even in solutions we have highly correlated structural changes.[26,45] Using Eq. (3) we can decompose the protein (solvent) motions to projections on the reaction coordinate. What is found is that the reaction coordinates in both the protein and solution will involve projections along the environmental coordinate. The difference is that the amplitude of the environmental change is smaller in the enzyme, reflecting smaller reorganization energy. In restating the discussion above, we note that studies that have used the DP method (see Ref. 2) have demonstrated that all that was done with the mode coupling picture amounts to expressing the reaction coordinate along a harmonic (or quasiharmonic) path. This provides a useful way to decompose the reorganization energy, but that the catalysis is actually associated with the reorganization energy and not with any time dependence of coherent modes.

Incorporating the spectral density in the diabatic (small coupling) rate expression and using our unique picture of intersecting vibronic levels,[94] and then moving to the more rigorous picture of the autocorrelation of the time dependent EVB energy gap (Refs. 90 and 44, lead to the quasiharmonic rate constant. Starting from

$$k_{ab} = \sum_{mm'} k_{am,bm'}\exp\{-E_{am}\beta\} \Big/ \sum_m \exp\{-E_{am}\beta\}, \tag{4}$$

where E_{am} is the energy of the m*th* vibronic level of state "a." Following our derivation (see Refs. 44 and 90), one obtains

$$k_{am,bm'} = \left|H_{ab}/\hbar^2\right| \sum S^2_{mm'} \times \int_{-\infty}^{\infty} \exp\left[(i/\hbar)\left\langle \Delta\varepsilon_{bm',am}\right\rangle + \gamma(t)\,dt\right], \quad (5)$$

$$\gamma(t) = -(i/\hbar)^2 \int (t-t')\left\langle \Delta\varepsilon(0)\,\Delta\varepsilon(t')\right\rangle_a dt'.$$

Here $S_{mm'}$ is the Franck-Condon factor for transition from m to m' and H_{ab} is the off-diagonal electronic matrix element of the EVB Hamiltonian, where u is given by

$$u = \varepsilon_b - \varepsilon_a - \langle \Delta\varepsilon_{ba}\rangle_a. \quad (6)$$

In the high temperature limit, we obtain

$$k_{am,bm'} = \left|H_{ab}S_{mm'}/\hbar^2\right| \left(\pi\hbar^2/k_BT\beta\right)^{1/2} \exp\left\{-\beta g^{\ddagger}_{mm'}\right\}. \quad (7)$$

The activation free energy in Eq. (7) can be approximated by

$$g^{\ddagger}_{mm'} \approx \left[\Delta G^0 + \sum_r \hbar\omega_r\left(m'_r - m_r\right) + \lambda_{cl}\right]^2 \Big/ 4\lambda_{cl}, \quad (8)$$

where λ is the "solvent reorganization energy" and ω_r is the indicated vibrational frequency.

The above treatment is only valid when $H_{ab}S^2_{mn}$ is sufficiently small. However, in cases of PT and HT processes, H_{ab} seems to be far too large to justify the above approximation but the vibronic treatment may give a very useful insight on the dependence of the rate constant on the donor acceptor distance, in relatively large distances when the diabatic approximation is valid.

Interestingly, Klinman and coworkers (e.g., Ref. 95) who used a similar formulation, like other theoreticians (with the exception of us), could not relate $g^{\ddagger}_{mm'}$ to $\Delta E^{\ddagger}_{mm'}$ (all the derivations of Marcus' relationship, with the exception of Warshel's energy gap treatment,[44,90] could not formulate consistently the relationship to the vibronic free energy) and could not determine the origin of the catalytic effect they were observing, which turned out to be a reduction in the electrostatic contribution to $\gamma(t)$. This led the authors to propose a dynamical coherent mode, although they could not determine its origin or how it reduces the barrier. This, in part, led to subsequent discussions about mode coupling and promoting modes (for debates, see Refs. 2, 96, and 97), which could not be identified in Eq. (7), although non-Boltzmann population of the frequencies in this equation would clearly have been considered as a dynamical effect.

In discussing the mode-coupling proposal, it is useful to also mention the work of Miller and coworkers,[76] who used EVB calculations and basically obtained the same results as those obtained by us.[98] In particular, their work found only very small role for non-local vibrational dynamics in enzyme catalysis.

The discussion of dynamical effects frequently includes the implication that compressive modes contribute to catalysis (this idea includes the tunneling proposal that will be considered in Section VIII). An example of this is given in Ref. 93, where it was suggested that the catalytic reaction of purine nucleoside phosphorylase involves protein modes that reduce the barrier height by as much as 20%, by compressing the reacting fragments. However, the enormous catalytic contribution of these modes was not directly calculated and any verification of such proposal must involve calculations of the barrier height. Additionally, it is crucial to realize that the same compressive mode exists in the solution reaction (see further discussion in Ref. 2).

While we have pointed out some major concerns with such studies before,[2] we also point the reader to an independent recent analysis,[99] which pinpoints more technical issues and yet concluded (in contrast to arguments that presumed that there are problems with transition state theory) that, with the proper corrections, the corresponding rate constant is fully valid for enzymes.

Some attempts to support the idea of compressive modes have been presented based on high-pressure experiments.[100] Unfortunately, not only do calculations of the actual change in barrier upon increases in pressure appear to be negligible relative to the catalytic effect, but also, the only real evidence for the compressive effect is the change in tunneling (see Section VIII B), and the observed effect is such that under high pressure, the donor-acceptor distance *increases rather than decreases*. This issue is analyzed in further detail in Section VIII B and Ref. 97.

It is also useful to point out that one of the systems where the compression idea has been promoted is catechol O-methyltransferase (COMT), where it has been proposed that mutation of residue Y68 to A reduces the rate of the enzyme due to an increase in the effective donor-acceptor distance.[101] Unfortunately, the geometric assertion was found to be problematic (see the discussion in Ref. 102), but, more importantly, actual calculations of the activation barrier due to the mutation[102] established that here we are observing a change in the electrostatic preorganization, rather than any NAC, compression, or entropic effect (see here also Ref. 103). Our point is not the issue of who is right or wrong, but rather only what actual calculations of the catalytic contributions (rather than indirect interpretations of the experimental results) can tell us about the origin of the change upon mutation.

At this point it is important to address a new twist in the argument about the NAC effect in COMT, where Klinman and coworkers[104] introduced a problematic analysis that is likely to confuse the uninitiated reader. More specifically, the authors of Ref. 104 simply misrepresented our analysis, claiming that "*In a recent computational study of COMT and its Tyr68 mutant (Y68A), the empirical valence bond methodology was also unable to provide a physical basis for the KIEs, dismissing these effects as circular interpretations of experimental results*." Of course, this allegation overlooks both our actual analysis[102] and the issue of the presumed catalytic effect from NACs. That is, our work[102] had not attempted to reproduce the KIE, since we have reproduced this effect in similar cases (and in fact we were the first to do so while considering the actual enzyme, see, e.g., Ref. 102 and an *earlier paper in* Section VIII). Rather, the issue was and has been the *reproduction* of the actual changes in the observed catalytic effect by a realistic molecular model (such as by QM/MM calculations), followed by establishing the

origin of these changes (which is only possible to do if they can actually be reproduced). Note that our EVB simulations model has reproduced the catalytic effect and its change upon mutation, and pinpointed the energy contributions that led to these changes for numerous systems (see Section X), including some of the most careful studies of the KIE in DHFR. Such accomplishments go back to our early studies of the NAC effect (e.g., Ref. 105), and is completely different from the attempts of Klinman and co-workers, who never reproduced the change in the activation barriers, let alone determine the reasons for such changes. Of course, the EVB has been used by now by many research groups in quantitative studies of enzymatic reactions and in evaluations of KIEs. Note in this respect that even reproduction of the changes in the reactant state structure (e.g., as was accomplished in Ref. 104) cannot tell us much about the origin of changes in the activation barrier. Showing that the KIE (or the NAC) has some correlation with the observed change in catalysis does not and cannot tell us much about the origin of these changes, as the issue is not reproducing the KIE, but rather analyzing the implied energetics of the NAC catalytic effect (our point on the circular analysis was related to the discussion of the correlation with the KIE instead of actually obtaining the barrier change). As we and others have shown, in quantitative analyses that reproduced the catalytic effect of the mutations and also reproduced the observed structural changes, proper study of the NAC contribution must involve free energy analysis that has very little to do with the observed KIE. Our papers actually reproduced the catalytic effect and established its electrostatic origin (see Refs. 102 and 105), something which has not been accomplished by Klinman and co-workers.

The analysis of Ref. 104 contains further major misleading aspects, in promoting GPU QM/MM studies that explored the geometrical distribution in the RS region. In doing so, it is implied that one needs a very large QM system (and thus presumably studies with smaller regions are invalid). This presumption overlooks the fact that comparing QM/MM calculations in enzyme to those in solution allows one to use a much smaller QM system than in calculations that do not involve such a comparison. In addition, the authors ignored the fact that using polarizable force fields allows one to use a smaller QM region. The correct way to judge what is needed in accurate modeling is to calculate and reproduce the observed activation barrier, and to then determine what the actual reason for the observed changes is (which was obviously not done in Ref. 104). In other words, although being able to include a large QM region in the simulations is a significant advantage, it does not help in analyzing the NAC effect. Here, the implications of Ref. 104 that previous calculations must have been incorrect is not only misleading but also overlooks the fact that the GPU QM/MM calculations were still unable to reproduce the activation barrier or even the RS free energy along the NAC coordinate. Without calculating the catalytic effect, it is simply impossible to figure out what the origin of the NAC effect is, and even reasonable calculations do not reproduce the true origin of the NAC effect [see Ref. 105] arriving at the common error of assuming that it reflects RS repulsion, whereas in most cases it reflects the fact that the active site stabilization of the TS also results in some RS compression. At any rate, once the observed catalytic effect of mutations is quantitatively reproduced (as was done by our study), the model can then be applied to the exploration of the reason for the overall effect, which is apparently electrostatic.[105] Attributing this effect to tunneling is not only inconsistent, but has also never been established by showing how the tunneling provides the correct energetics. Finally, readers who are still willing to believe in the NAC proposal after these arguments are welcome to do so.

In summary of this section, we conclude that, in contrast to the implications in the discussion of the landscape of Refs. 41, 106, and 107, the enzyme modes are *always* coupled to the chemical coordinate, but this coupling is not dynamical in its nature.

VI. DYNAMICAL EFFECTS AND FREE ENERGY LANDSCAPES

The complex nature of the free energy landscapes of enzymes has attracted significant interest,[1,108–113] and led to proposals that this complexity can rationalize the rate acceleration by enzymes. In fact, we have previously discussed this issue[1,114,115] noting that the complexity of the landscape is not the reason of the catalytic power. In fact, as we will outline in this section, the complexity is itself the problem, rather than the solution to the problem.

Well-defined questions about the relationship between the landscape and the catalytic power of enzymes were first formulated in our work[113] (in the form of diagrams such as in Figure 5). However, some issues about the impact of the shape of the landscape on the rate acceleration remain unresolved, including the (unjustified) view that the complexity of the landscape presents a dynamical factor. This has been argued, for example, in Ref. 41 which suggested that the landscape sampling is somehow a dynamical effect, although sampling of the thermodynamic landscape has little to do with dynamics. Of course, correct analysis of the activation free energy cannot be achieved without proper conformational sampling, and such sampling has been a key element of our EVB treatment from its early MD implementation, starting already in the early

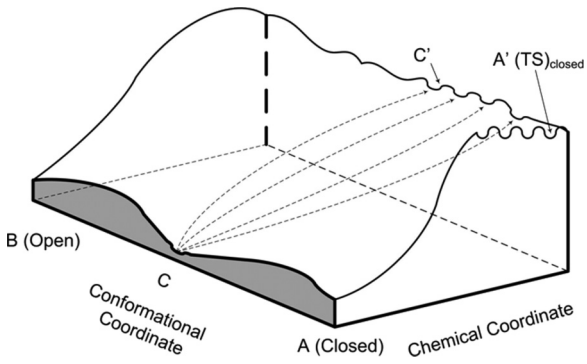

FIG. 5. A schematic representation of the free energy landscape as a function of the conformational and chemical coordinates in a reacting enzyme. The figure depicts trajectories across the conformational coordinate and a continuation of this trajectory along the chemical reaction coordinate. Reprinted with permission from Kamerlin, S. C. L., Mavri, J., and Warshel, A., "Examining the case for the effect of barrier compression on tunneling, vibrationally enhanced catalysis, catalytic entropy and related issues," FEBS Lett. **584**, 2759 (2010). Copyright 2010 Federation of European Biochemical Society.

1980s (see, e.g., Refs. 27 and 45). Of course this sampling gives entropic contributions, which again have nothing to do with dynamics, but rather are a reflection of the available configurational space.

Experimental studies[116,117] of the enzyme chorismate mutase (CM), which catalyzes the conversion of chorismate to prephenate, pointed out towards a relationship between the folding landscape and the enzyme activity. The nature of these findings was explored by our simulation study,[114] which determined the activation barriers for different conformational regions. However, works that have not attempted to explore the actual landscape led to the proposal that the search of the landscape leads to catalytic effect.[118] However, the search on the landscape is the natural behavior of any system that tries to pass the TS with the corresponding Boltzmann probability. Of course, cases where there are several passes across the TS ridge,[2] we have to consider these paths in evaluating the activation entropy. Thus, the reaction landscape can contribute to catalysis if there is a much larger configurational space at the TS than in the RS. For example, analysis of the catalytic landscape of DHFR (and its mutants) has been reported in Ref. 73. This work illustrated that, in contrast to the implications of Ref. 40 and others, the changes in the topology of the free energy landscape can lead to changes in catalysis by energetic rather than dynamical effects. More specifically, some of the changes in the catalytic effect of DHFR upon mutation are due to the entropic effect of having more crossing ridges at the TS than at the RS, or vice versa (see the analysis in Ref. 73). Obviously, this is a thermodynamic effect, as the configurational space is not dynamical. As discussed in Ref. 2, such a well-defined effect should not be described as an "entropy funnel" (see Figure 9 of Ref. 119). In brief, unless such funnel proposals are formulated by a well-defined physical description that can be studied by physically based method, it is not possible to assign to them significant dynamical contributions to catalysis. Obviously, the nature of the activation entropies has to be explored and this can be accomplished by our restraint release approach.[120,121]

At this point, we note that in analyzing possible landscape effects, it is useful to also explore the possibility that many trajectories start from the same restricted RS and then pass many points at the TS.[2] Now, contrary to arguments put forth in Ref. 111, this is not necessarily a fundamental problem, nor does it provide a catalytic advantage. For example, in the case presented, we have an entropic advantage by having in the TS a wider hyperspace, in the direction perpendicular to the reaction coordinate than in the TS. In this case, however, we might have to consider the extra dynamics effects, associated with jumping between the different states across the TS ridge. Thus, we may have to consider the time dependent kinetics of moving between A' and C' of Fig. 5. If, for example, the barriers between the valleys that go from the RS to the manifold at the TS are small we might have to introduce some dynamical corrections.

VII. FLEXIBILITY AND DYNAMICAL EFFECTS

Many works have proposed that enzyme rate acceleration is related to their flexibility.[14,15,122–126] This idea was seemingly supported by studies of thermal adaptation.[14,15,122,123] That is, it was thought that since thermophilic (*Tm*) enzymes that function at highly elevated temperatures are more stable and have higher catalytic power than the corresponding mesophiles (*Ms*) variants, and since these enzymes have lower catalytic power than the *Tm* enzymes, it has been concluded that reducing the dynamical motions decreases catalysis.[14,127,128] This proposal was examined by simulations of DHFR,[98] where it was found that the chemical coordinate and the folding coordinates are actually perpendicular and that the catalysis is determined by the chemical reorganization energy (for more discussion, see Ref. 2). Overall, it was found that the catalysis requires less flexibility rather than more flexibility.

This is supported by recent studies[129] of interfacial activation of serum paraoxonase 1 by association with high-density lipoprotein (HDL), where it was demonstrated that the activity stimulation of this enzyme upon association with HDL is due to rigidification of a hydrogen bonding network that spans over 20 Å from the surface of the protein through to the catalytic core of the enzyme, and keeps the key catalytic residues in place. This work also discussed the "dark side" of excessive dynamics in enzyme design studies.

Finally, Åqvist and coworkers have recently reported some very instructive studies that explored the action of cold-adapted enzymes,[130] and demonstrated that the temperature adaptation is controlled by the entropic effects of residues on the protein surface, which in turn leads to entropy-enthalpy compensation (and has nothing to do with dynamical effects).

VIII. TUNNELING AND CATALYSIS

Studies of isotope effects in enzymatic reactions (e.g., Refs. 131 and 132) have provided exciting information about nuclear tunneling effects in such reactions (e.g., Refs. 118, 106, 107, and 133). This information has led to the interesting proposal that nuclear quantum effects (NQM) contribute significantly to the catalytic power of enzymes, and that these contributions involve dynamical effects. The validity of these proposals will be examined in detail below.

A. Tunneling contributions are similar in enzymes and in the corresponding solution reactions

Klinman and coworkers have provided clear evidence of NQM in several enzymes,[15,134–138] and these findings have frequently been interpreted as evidence that the width of the barrier for tunneling is narrowed by particular vibrations.[139] The identification of large tunneling was used to argue that NQM is important for catalysis (see, e.g., Refs. 95 and 140). Unfortunately, however, as was pointed out in our previous works,[1,59,141,142] similar contributions also appear in the reference solution reaction.

Since it is frequently hard to measure the reference solution reaction, we developed the quantum classical path (QCP) method[143] and established that the NQM are similar in enzymes and solution reactions. Interestingly, works that have adopted our QCP strategy have found very little, if

any, catalytic effect from tunneling. For example, Ref. 144 studied differential NQM contributions in the catalyzed and uncatalyzed proton transfer reactions of nitroalkane oxidase, and calculated an 8.3 kcal/mol barrier reduction in the enzyme catalyzed reaction compared to the corresponding uncatalyzed reaction in solution (see Table 1 of Ref. 144), which is equivalent to a 10^8-fold rate acceleration. Of this tremendous barrier reduction, only 0.6 kcal/mol was calculated to be due to tunneling effects (<10-fold) and thus although very slightly larger tunneling contributions were observed in the enzyme than in solution, the contribution to the rate-acceleration is still negligible.

B. The fatal flaw of the catalytic tunneling proposal

The main argument in favor of the tunneling idea has been that the enzyme compresses the donor acceptor distance creating a narrower potential and larger tunneling.[135,140,145–148] However, our studies and that of others[143,149] established that the NQM effects *decrease* rather than increase due to compression. Apparently, when the distance between the donor and acceptor is sufficiently compressed, the mixing between the two *electronic* states makes the adiabatic surface very flat, so that the tunneling effect decreases (see Ref. 97 and Figure 6).

In fact, our analysis established that the increase in NQM (and KIE) corresponds to reduction in catalysis (since the barrier increases when the donor acceptor distance increases (see, e.g., Ref. 150 for discussion)). In our view, one of the most profound problems of the catalytic tunneling proposal is the fact that, after we have pointed out the fundamental anticatalytic picture in the original tunneling effect (i.e., the fact that compression reduces the tunneling rather than increasing it), there has been no further comment on this fact from supporters of the catalytic tunneling idea.

C. The temperature dependence of the KIE

The temperature dependence of the classical and NQM effects, as manifested by Arrhenius plots and the corresponding KIE, has been a topic of great interest.[61,95,106,151] This interest reflected in part the assumption that the deviation from linearity of the Arrhenius plots reflect dynamical effects.[95,152]

It was argued that the temperature dependence of the KIE means that the tunneling is thermally activated and thus contributes to catalysis (e.g., Refs. 107 and 153). Now tunneling can clearly be thermally activated but the same thermal activation occurs in solution.

Reference 15 found out that thermophilic alcohol dehydrogenase (ADH) has a nonlinear Arrhenius curve and

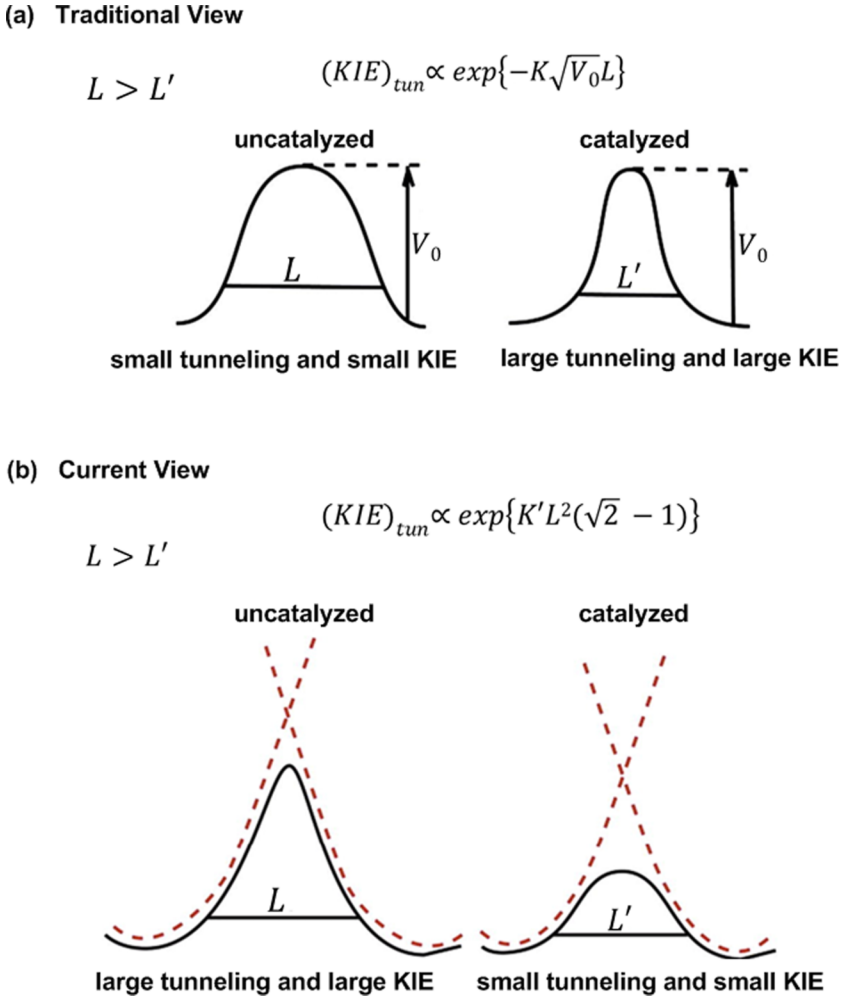

FIG. 6. Illustration of the (anti-) catalytic effect of increasing the donor-acceptor distance dependence on the NQM effects in hydride transfer reactions. Panel (a) describes the traditional model, in which NQM effects increase upon compression of the donor-acceptor distance, and assumes that enzyme catalysis involves an increase in tunneling due to the compression effects. (b) Demonstrates our view, based on extensive simulations and theoretical analysis, that the NQM effects actually *decrease* when the donor-acceptor distance decreases, as compression of the donor-acceptor distances causes the barrier to collapse and makes the process fully classical (see the discussion in Ref. 143). Reprinted with permission from Liu, H. and Warshel, A., "Origin of the temperature dependence of isotope effects in enzymatic reactions: The case of dihydrofolate reductase," J. Phys. Chem. B. **111**, 7852 (2007). Copyright 2007 American Chemical Society.

interpreted this observation as supporting a contribution to k_{cat} from vibrationally enhanced tunneling at higher temperatures. Although the authors attributed this finding to dynamical effects, we have noted[27,120,1] that probably most of the effect, and, clearly, its largest classical contribution, is an entropic effect that can be rationalized by considering the expected interactions of the solute with its surroundings (as a result of the change in polarity of the reacting atoms, see Ref. 97).

Significantly, simulations of the NQM of DHFR[143] and lipoxygenase[59] have shown that the KIE increases when the donor-acceptor distance increases. Moreover, the temperature dependence of the KIE was found to mainly reflect the temperature dependence of the donor-acceptor distance. Thus, the trend in temperature dependence indicates that the tunneling is anti-catalytic (see Refs. 143 and 150). Overall, we concluded that there is no consistent experimental evidence of NQM contributions to catalysis, and all consistent computational studies have not found any support for the existence of significant NQM contributions to catalysis. A concise and logically consistent review of the relevant considerations is given in Ref. 150.

IX. PREORGANIZATION IS THE KEY FACTOR IN ENZYME CATALYSIS

Although the importance of electrostatic contributions to enzyme catalysis is now widely accepted (for a detailed review, see Ref. 1), this idea was slow to gain recognition by the physical organic community,[154,155] due to the finding that such effects are small in solution reactions. Similarly, the seminal work by Jencks[156] overlooked the electrostatic TS stabilization and proposed desolvation and RS destabilization. Thus, it was not until the 1976 work of Warshel and Levitt[51] that the major role of electrostatic effects in catalysis was clearly demonstrated. However, since 1976, it has become quite clear that electrostatic effects are central to catalysis[1] (and more recently also to enzyme functional evolution[157]). The nature of the electrostatic stabilization has been recognized first in Ref. 3 and appears to reflect a polar preorganization.[3] This effect reflects the fact that the solvent dipoles have to pay reorganization energy while rotating toward the TS charges, whereas the enzyme dipoles are partially oriented toward the TS.

Apparently, the important concept of catalytic preorganization has not been fully recognized (reflecting in part its non-intuitive nature) and this might be in part the reason for the acceptance of inconsistent proposals such as the dynamical proposal. This possible misunderstanding is discussed in Ref. 2, and is possibly a result of problematic interpretations. Some examples of this include Ref. 158, that not only renamed the preorganization concept to describe something fundamentally different, but even more dangerously, incorrectly used the terminology of the original preorganization concept in doing this (see discussion in Ref. 2). Another major example of the misunderstandings of the preorganization concept is ketosteroid isomerase (KSI), which uses an oxyanion hole to stabilize an anionic intermediate during the isomerization cycle, and thus provides perhaps one of the best illustrations of the preorganization effect. We have discussed this system in great detail in our previous review,[2] and therefore will not repeat the discussion here but rather point readers to that work. However, we would like to point out that the view that KSI catalysis is largely due to electrostatic TS stabilization was also strongly supported by the recent groundbreaking experimental work of Boxer and coworkers,[159] as well as by recent computational study of DHFR by Moliner and coworkers.[55] Similarly, although Ref. 26 did not explicitly focus on the reorganization energy, it provides additional support to our concept.

X. SOME BACKGROUND ON THE COMPUTATIONAL APPROACHES

Simulations of enzymatic reactions require the use of multiscale,[160] and, in particular, hybrid QM/MM approaches.[161] Unfortunately, despite significant advances in this direction, we do not have fully rigorous simulation studies with both *ab initio* quantum region and full sampling that provide the proper free energy surface. In this respect we like to point out that using QM/MM surface with energy minimization does not provide correct way of exploring activation barriers in enzymes. Although the sampling problem can be reduced by using reference potentials,[161–164] it seems to us that even at present the EVB is the most effective way of exploring catalytic effects in enzymes. The effectiveness of this approach, when it is calibrated on the energetics of the reference solution reaction (e.g., Ref. 165) and then applied to the enzymatic reaction, has been recognized by many workers other than us (see, e.g., Refs. 87, 149, and 166–168 to name just a few examples). Perhaps the best indicator of its increasing popularity is the proliferation of recent reincarnations of the EVB approach under different names, such as those of Refs. 169 and 171 (see the discussion in Ref. 170). At any rate, the power of the EVB has been established in studies of the effect of long range mutations,[56] in evaluating entropic effects, in studies of NQM,[68] and in exploring benchmarks for enzyme design.[172–174]

XI. CONCLUSIONS

The assumption that enzyme dynamics is important for catalysis has gained popularity over the past two decades, and has been the subject of significant experimental and theoretical investigation. Unfortunately, the slow advances in reliable theoretical studies may reflect the slow progress in the belief that the origin of enzyme catalysis can be quantified and judged by computer simulations. It can also reflect the difficulties in following the preorganization concept, and the fact that alternative catalytic proposals (such as low-barrier hydrogen bonds and ground state destabilization[1]) have been very problematic and poorly defined, and thus harder to disprove for those who are not determined to have clear and unique definitions. In this work, we scrutinize the dynamical proposal and considered the most compelling arguments against this proposal and conclude that there are no consistent studies that found significant dynamical contribution.

At this point, it is useful to realize that theoretical approaches that support the dynamical proposals cannot be accepted unless they are able to reproduce the actual rates and their change with mutations. The examples given in our previous works[1,27] have provided pointers on how to assess theoretical studies of dynamical effects, such that readers who do not necessarily accept our conclusions are at least aware of what has been established through careful theoretical considerations. We truly hope that the examples above, combined with logical analysis, will lead readers to accept that at present we still cannot find a single consistent study that demonstrated that dynamical effects increase the rate constant significantly. Here, the requirement is that the study is able to evaluate the actual free energy landscape by sufficient sampling, and to actually reproduce the observed catalytic effect.

As can be seen from the discussion in the literature, the field is indeed controversial, perhaps in part because of the fact that much of the discussion of this topic has been very qualitative rather than using clear physical and logical concepts. Furthermore, the idea that protein fluctuations have a role in catalysis has been strongly supported by some of the most active workers in the field (see, e.g., Refs. 36, 38, 108, 112, and 175 among others for examples). This includes the *clear* suggestion[33] that there exists a "*pre-sampling of conformational substrates before catalysis that are harvested for catalytic turnover*."[33] Related arguments have been summarized in Ref. 2. Thus, the fact that some of the original promoters of the dynamical proposal argue now that dynamics does *not* actually contribute to catalysis[34] (see discussion in Ref. 176) may lead to significant misunderstanding.

In order to clarify our point of view, we put significant effort in establishing the need for defining the dynamical idea in terms of reaction coordinate and energy landscape. We also state that ideas like "landscape searches," "entropy funnels" as well as "promoting motions" (see Ref. 2) cannot account for the rate acceleration by enzymes. We point out that the best way to see the problems with the dynamical proposal is to formulate this proposal in a clear physical way. Without doing so we will end up with circular discussion. Having done so, and after covering probably *all* reasonable definitions of dynamics and catalysis, we concluded that there are in fact *no* significant dynamical contributions to enzyme catalysis.

At this point, it would be useful to comment further on the recent tendency to argue that the debate on the role of enzyme dynamics in catalysis can be boiled down to a simple semantic issue (see, e.g., Ref. 177). For example, in a notable instance, it was argued that controversy has erupted over "*experimentalists' usage of the term* "*dynamics*," *which has been met with rejection by theoreticians who assumed dynamics meant non-statistical motions*, *even though the experimentalists using the term* "*dynamics*" *obviously meant thermally equilibrated dynamics (as is evident from their use of* Eqs. (3) and (4). *Both* Refs. 158 and 2, *for example, assume statistical dynamics, but due to different terminologies (and because they are focused on different aspects of catalysis), many statements by these researchers appear to contradict one another*." Before providing a longer clarification, we note that one researcher suggested that dynamics contributes to enzyme-catalyzed reactions,[15,41,95,106,118,133] while the other claims that non-statistical dynamics, if they contribute to the rate at all, are not statistically different in solution versus enzymes.[2] As another example, the first researcher mainly focused on events of critical importance to biological systems, such as the one fold rate-enhancement and the fine-tuning of the system to reach its exquisite specificity and control, whereas the second researcher, on the other hand, mostly focused on the many orders of magnitude difference between catalyzed and unanalyzed reactions, rather than obsessing over to ±1 kcal/mol effects on the barrier height, which might be life or death for the organism, but has little bearing on the uncatalyzed reaction and in particular on the key question addressed here, namely the origin of enzyme catalysis. Additionally, arguments that both researchers see the nature of enzyme-catalyzed reaction in a similar way and only the titles are different, are problematic, as catalysis has no meaning without a well-defined reference state, and any vague implications of dynamical rate enhancements must show what such enhancements are relative *to*. That is, even if they defined as the activity of the native enzyme relative to its mutants, it must be shown that this is actually due to dynamical effects. However, no such demonstration has been provided.

At this point, it is also useful to expand about the above presumed semantic issues, because of the potential major confusing nature of the above statement. Here, we will first clarify its major misleading parts, for example, the quotation in italics above (describing the experimental and theoretical branches of the field) creates an artificial division between semantic versus substantial issues (e.g., Ref. 177). Additionally, the suggestion that experimentalists *always* meant "statistical dynamics" when discussing dynamics, while the theoretical community "objected to the non-statistical dynamics" may sound reasonable and consolatory, causing readers to tend to accept it. However, the argument is actually very problematic, because, most (if not all) of the experimentalists that worked on this issue supported the "real" dynamical proposal (in contrast to the questionable stochastic dynamics), where at most they could not define it, but it is completely clear that they meant a real dynamical effect (what is now called by some "active dynamics") and not just sampling effects. Clear examples of this have been documented in Ref. 2.

In addition, Klinman and coworkers (e.g., Refs. 95, 106, and 135) not only focused on catalysis by tunneling (which is problematic, as discussed in Section VIII), *but also promoted ill-defined real* "*active dynamical*" *effects, that do not, however, contribute significantly to catalysis* (*see below*). In particular, these workers used a formula that is similar to Eq. (8), but had several problems. Firstly, they overlooked the key points that lead to catalysis, not realizing what factors reduce γ (namely electrostatic preorganization). Additionally, their so-called Marcus-like formula is not based on Warshel's surface hopping treatment,[94] but rather on Kuznetsov and Ullstrup,[151] so it does not actually have activation *free* energies, and it misses the protein's entropic effects. Now, there seems to be a separation between passive dynamics, that presumably determines the activation enthalpy and active dynamics, which modulates the donor-acceptor compression modes (except for

the fact that compressive modes do not work, as outlined in Section V). However, this is still clearly an "active dynamics" proposal, even if we restrict our semantic argument to the active dynamics gating mode. It is therefore easy to see why Ref. 106 quotes and supports workers with such gating mode.[153] For the sake of argument, we would also like to point out that, already in 1999, Ref. 15 claimed that enzymes control vibrations, which is opposite to the equal partition needed in a stochastic model, and there is no single case with a correct prediction. That is, these works took an expression derived by others, with its own problem (diabatic and unclear free energy), and could thus involve some misdefinitions. However, clearly, the key proposal (tunneling) never worked, as is also the case for compression and dynamics.

After discussing one of the key attempts, claiming that the real dynamics proposal has never been made by the workers in the field, we can be more charitable and point out that Ref. 2 provides many examples of the very pronounced attempts to prove that enzymes work by dynamics (where it is very clear that the proposed dynamics has little to do with the newly defined "passive dynamics").

Finally, as to the presumed objection of theoreticians to the active dynamical (real dynamical) proposal, in fact most early theoretical works on the dynamical effect supported the real dynamical idea, and our group almost single-handedly focused on defining and excluding these proposals. Overall, since what determines catalysis is the activation free energy (which may be evaluated by Monte Carlo sampling that does not reflect any dynamical contributions), all the references to major dynamical control of catalysis have been with a strong belief that dynamics is important, rather than simple semantic confusions.

Another presumed general agreement in the field is the compression idea. Here we can repeat the clarification that Ref. 100 proposed the problematic idea of compression by dynamical vibrations (and also promoted this idea incorrectly as being consistent with experiments) while it is also presented as being identical to our findings.[102] Unfortunately, it is hard to accept the argument that both works meant the same. In fact, it is not so complex to see that Ref. 102 simply contradicts Ref. 100.

Recent years have also seen repeated implications that enzyme dynamics can be harnessed for enzyme design (e.g., Refs. 41 and 178), but this seems extremely unlikely, as was eloquently demonstrated by Gobeil *et al.*[179] and discussed by Tokuriki and Jackson,[180] that engineered changes in the millisecond mutations of mutant TEM-1 β-lactamase do not significantly affect substrate turnover. This mutational robustness has implications for protein engineering and design strategies. In addition, there is a "dark side" to excess enzyme dynamics, in that floppiness impairs catalytic efficiency and promotes futile encounters.[129,181] Therefore, if anything, rational design efforts are best directed towards reducing excessive conformational flexibility in *de novo* enzymes.

In summary, we would like to emphasize that the dynamical idea has been finally explored by theoretical studies that can stimulate the coupling between the conformational and chemical motions (e.g., Refs. 2, 55, 74, 76, 99, and 182), and these studies have found that the conformational fluctuations do not affect the chemical rate constant. In fact, our view about protein dynamics is increasingly shared by other eminent workers in the field. On the other hand, we are not aware of any experiment that has actually established a dynamical coupling between the conformational and chemical coordinates of an enzymatic reaction. Although verifying this finding still presents a challenge for future experimental and theoretical studies. However, the only direct consistent analysis in the field determined that the chemical step does not remember the conformational motion, and thus that dynamics does not play an important role in catalysis,[80] and is unlikely to provide the future of understanding enzyme catalysis.

Note, also, that many of the works that have supported the dynamical hypothesis have not at all addressed the concerns that were raised in our analysis. Furthermore, negligible effects (e.g., minor changes in NQM) have been frequently presented as major catalytic factors. In fact, no consistent calculation that actually supported and quantified the dynamical proposal has been presented. Of course, one may assume that no calculation can provide any reasonable result for such catalytic contributions, but as should be clear from this perspective, we do not share this view. We would like to also point out that this review was written with the aim of providing readers with sufficient examples and different aspects, provided in a logical framework, to allow them to make their own mind up about the likelihood of significant dynamical contributions to catalysis.

As mentioned above, it is interesting to note that recent works (e.g., Refs. 183 and 184) show the emergence of more agreement with our view including from those who originally opposed it,[41] but it would be unjustified to attribute this ongoing paradigm shift to merely semantic consensus. That is, as far as we can judge, the dynamical proposal has been problematic on any possible level, and trying to now formulate it with correct concepts (e.g., Ref. 41) seems to have little to do with the dynamic concept that was actually introduced and implied by its original proponents. For example, going back to the stochastic Marcus-like model,[41] and the confusions mentioned in the Introduction, the only real confusion that we can see is that before our microscopic work,[94] it was not clear (in light of the use of macroscopic models) if the term that expresses the Marcus activation barrier should include the entropic contributions. It was also not clear that, in most enzymatic reactions, we are at the adiabatic limit, so that the TST pre-exponential holds.

Throughout this paper, we have 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 in defining the opposing views. That is, without clear-cut definitions, it is impossible to conduct scientific discourse. Similar problems have arisen in cases such as the confusion between the non-zero projections of protein normal modes on the chemical reaction coordinate to dynamical effects, where we have a time-dependent memory of the motion in the non-chemical direction while passing the chemical barrier. Of course, this means that "stochastic dynamics" should not be used to define the dynamical proposal. In fact, suggesting that the arguments on the dynamical proposal should not include discussion of

non-statistical proposals, as implied by Ref. 41 is basically equivalent to arguing that there never was a real point of disagreement.

In conclusion, while we agree that it is reasonable to use Ockham's razor to exclude the dynamical proposal,[183] however, we also believe that basically using the "inductive" approach of showing that the proposal does not work for any current test case (as we have done here) is the best way to establish that enzyme catalysis is not due to dynamical effects.

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