Catalysis by dihydrofolate reductase and other enzymes arises from electrostatic preorganization, not conformational motions

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The proposal that enzymatic catalysis is due to conformational fluctuations has been previously promoted by means of *indirect* considerations. However, recent works have focused on cases where the relevant motions have components toward distinct conformational regions, whose population could be manipulated by mutations. In particular, a recent work has claimed to provide *direct* experimental evidence for a dynamical contribution to catalysis in dihydrofolate reductase, where blocking a relevant conformational coordinate was related to the suppression of the motion toward the occluded conformation. The present work utilizes computer simulations to elucidate the true molecular basis for the experimentally observed effect. We start by reproducing the trend in the measured change in catalysis upon mutations (which was assumed to arise as a result of a "dynamical knockout" caused by the mutations). This analysis is performed by calculating the change in the corresponding activation barriers without the need to invoke dynamical effects. We then generate the catalytic landscape of the enzyme and demonstrate that motions in the conformational space do not help drive catalysis. We also discuss the role of flexibility and conformational dynamics in catalysis, once again demonstrating that their role is negligible and that the largest contribution to catalysis arises from electrostatic preorganization. Finally, we point out that the changes in the reaction potential surface modify the reorganization free energy (which includes entropic effects), and such changes in the surface also alter the corresponding motion. However, this motion is never the *reason* for catalysis, but rather simply a reflection of the shape of the reaction potential surface.

The enormous catalytic power of enzymes has been attempted to be rationalized by several proposals. Here, we would like to focus on a specific proposal that appears to be gaining significant support. Namely, there exists a long-standing assumption that enzyme dynamics and flexibility are important to the chemical step of catalysis (see, e.g., refs. 1–4 and references cited therein). This hypothesis has emerged in several forms, ranging from the assumption that enzymatic catalysis can be linked to lid closures upon binding (e.g., ref. 5) to more recent studies (6, 7) that considered the effect of modifying the accessibility of conformational states separated by relatively small structural differences. It was then argued that the observed changes in the rate of the chemical step upon mutations that appear to prevent the flexibility of the active-site residues could be interpreted as evidence for a dynamical coupling to catalysis. This proposal is particularly well defined in a recent study (6) that focused on dihydrofolate reductase (DHFR). That is, ref. 6 demonstrated that the N23PP, S148A, and N23PP/S148A mutants of DHFR have more limited conformational flexibility than the WT and cannot access the occluded (OC) conformation from the closed (CL) conformation, which is available to the WT product ternary complexes. The above mutants also show a reduction in the rate constant of the hydride transfer step (k_{hyd}). Additionally, the authors noted that the structures of the active sites of the WT and mutants are very similar [though a change in donor-acceptor (D-A) distance from 3.3 to 2.9 Å was incorrectly considered by the authors as being "indistinguishable"], even though k_{hyd} for the various mutants are clearly different. This deduction led to the assertion that the structures are "identical," and thus that the electrostatic preorganization must also be identical. In other words, the observed reduction in k_{hyd} (which, even for the "worst" mutant, is actually a very small reduction of a factor of about 16 which only corresponds to a change of approximately 1.7 kcal mol^{-1} in the activation free energy) was attributed to a restriction of the conformational fluctuations of the active-site residues, and not to the logical explanation that the mutation changed the relative barrier height of the chemical step. Thus, it was argued that the mutations provide a dynamical knockout by decreasing the millisecond timescale fluctuations of residues in the active site, which were assumed to probably be in part responsible for promoting the hydride transfer. This knockout was assumed to lead to the corresponding decrease in k_{hyd}.

Because neither the precise nature of the aforementioned active-site fluctuations nor the coordinate on which they supposedly operate were actually clearly defined in ref. 6, we have taken special care to provide a generalized argument. Nevertheless, it would appear straightforward that the simplest and most logical candidate for the assumed flexibility coordinate corresponds to the active-site fluctuations in the direction of the path from the CL to OC conformations. Such a definition does not necessarily mean moving completely to the OC state, but only moving in its direction (see *Flexibility and Conformational Dynamics*). Additionally, it should be highlighted that the implication that the S148A mutant is almost as catalytically active as the WT (despite the blockage of the motion in the OC direction) is an example of the problems with discussing an undefined flexibility coordinate.

It seems to us that the above-mentioned analysis may involve a misunderstanding of the nature of reorganization energy (see also ref. 8). The underlying problems may arise from the incorrect assumption that electrostatic preorganization can be determined from examining experimentally based X-ray or NMR structures. Unfortunately, converting structural information to the corresponding reorganization energy can only be quantified by microscopic approaches that were introduced by our group for studies of electron transport (e.g., ref. 9). For these approaches, it is essential to have the X-ray structures of the reactant and product, or, in some cases, the structure of the reactant (or product) complexes, when the substrate is fully nonpolar with zero residual charges. In contrast to the implications of ref. 6, it is essentially impossible to determine the electrostatic contributions to the free energy from just viewing the structure, and this fact is easily

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demonstrated in the present work. This point would also be clear by simply trying to determine the pK_a of a given residue without an energy-based approach, from the X-ray crystal structure alone.

At any rate, because ref. 6 clearly argues that there exists coupling between conformational fluctuations and the chemical step of catalysis, an in-depth analysis of the corresponding assertions would provide further clarification of common misunderstandings of enzyme catalysis. For example, we will address the argument that the depressed hydride transfer rate in the DHFR mutants presented in ref. 6 compared to WT DHFR arises out of an inability to sample higher-energy conformational substates. We will also emphasize the fact that the rate constant has little to do with the availability of fluctuations on different timescales, but rather with the Boltzmann probability of reaching the transition state. Additionally, we will address the fact that the repeated discussion of flexibility rather than the reorganization free energy seems to focus on the wrong issue. Finally, we will also clarify that altering the flexibility along the conformational coordinate can only lead to minimal changes in the activation entropy, which do not correspond to dynamical effects (see *Entropic Contributions are Free-Energy Contributions*).

With the aforementioned perspective in mind, we will provide a quantitative analysis of the intriguing findings presented in ref. 6. Once the observed findings have been reproduced by unbiased simulations, we will proceed to determine the source(s) of the depressed catalytic effects of the relevant mutations, and demonstrate that the reduction in the catalytic effect is entirely due to changes in the electrostatic reorganization energy and *not* to dampening of millisecond conformational dynamics at the active site.

Analysis

Background. The role of the electrostatic reorganization energy in determining the activation free-energy barrier has been discussed in detail elsewhere (10, 11) and is briefly illustrated in Fig. S1, which shows two different ways the activation free-energy barrier can be reduced. In the first option (Fig. S1*A*), the position of the minimum of the product free-energy functional is shifted, which results in a lowering of $\Delta g^{\ddagger}$ because of a reduction in the reorganization free energy along the chemical coordinate. In the second option, $\Delta g^{\ddagger}$ decreases due to the reduction of the overall free energy of reaction (ΔG_0), as a result of an increase in the solvation of the product state (PS) charges or decrease in the solvation of the reactant state (RS) charges. Here, the catalytic effect is also associated with the reduction of the reorganization free energy, but this time the effect is due to a change along the solvation coordinate rather than the chemical coordinate.

In general, there is a distinction between the reorganization free energy along the chemical coordinate (λ) and the one along the solvation coordinate (λ'). In the case of the DHFR mutants examined in our previous studies (12), the reorganization effect could be represented as in Fig. S1*A*. However, in the more recent case (6), the mutations lead to a reduction of ΔG_0 and the analysis is more complex (see below).

At this stage, it is also important to ask what might be implied by "active-site dynamics" in ref. 6. That is, our starting point is a proposal that cannot be analyzed without a clear definition, because enzymes move thermally all the time and are not static entities (see ref. 13). However, as long as the chance of moving from the RS to the PS is controlled by the corresponding Boltzmann probability, there is no need to invoke any dynamical effects. Nevertheless, ref. 6 uses the fact that NMR found a correlation between the millisecond motions of the active-site residues and k_{hyd} to circumstantially support the dynamical idea. Now in order to explore this proposal, and in the absence of observables capable of separating between the motions along the reaction coordinate and the orthogonal conformational directions, we are forced to resort to logical deduction to understand what is meant by the assertion of ref. 6. Here, we are left with no choice but to conclude that ref. 6 must be referring to the proposal that the dynamics in the *conformational* direction helps catalysis, and we have examined this issue in greater detail in the section titled *Flexibility and Conformational Dynamics*. Finally, we note that it is also important to avoid confusing the catalytic cycle with the catalytic step (chemical catalysis), as discussed in the *SI Text*.

Examining the Origin of the Differences in the Catalytic Effect. The task at hand is to explore the origin of the small difference in the catalytic effect of the WT, and the N23PP/S148A and S148A mutant forms of DHFR (see Fig. 1). The ability to explore relatively small mutational effects demands a stable simulation approach that allows one to reliably sample the enzyme landscape. At present, perhaps the most effective approach for doing this is the empirical valence bond (EVB) approach (the details of which are given elsewhere, see, e.g., ref. 11), which has already been used in studies of DHFR (12, 14, 15) and is being used in this study. Our previous EVB studies (12) have established the correlation between the activation barrier and the reorganization free energy plus work function, which is shown in Fig. S2. Here, however, we are dealing with the different mutants presented in ref. 6 and with smaller effects.

As a starting point, we evaluated the catalytic effect of the WT and reproduced it, as in our earlier works on this system (12). Subsequently, we examined the activation barrier in the N23PP/S148A and S148A mutants and obtained the results summarized in Fig. 2 and Table S1. As is clear from the table, we reproduced the observed trend of the effect of the mutant, and thus established that the effect of the mutation is most likely due to a free-energy change and not to esoteric dynamical effects. That is, although the observed effect is very small (and cannot be *exactly* reproduced by any existing simulation approach), we established that the mutation leads to a change in barrier that accounts for the observed trend in k_{hyd}. This finding, which cannot be obtained by any current experimental strategy (because it is impossible to experimentally dissect the contributions of the mutation to the altered catalytic efficiency), proves that the implication of ref. 6 that the surface does not change is incorrect.

Having reproduced the depression in the catalytic effect of the mutants, we examined the origin of the corresponding free-energy change by evaluating the free-energy functionals (11), Δg_1 and Δg_2, which provide the microscopic equivalent of the Marcus parabolae. The corresponding results are shown in Fig. S3. As seen from the figure, the intersection of the two parabolae ac-

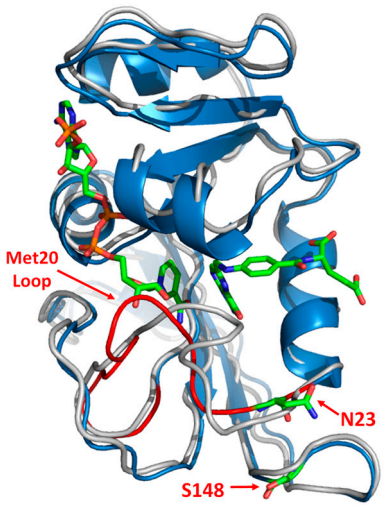

Fig. 1. A superimposition of WT DHFR in the closed [blue, from Protein Data Bank (PDB) ID code 1RX2] and occluded (gray, from PDB ID code 1RX4) conformations. The mobile Met20 loop (red, residues 9–24) and the sites of mutation (N23 and S148) are indicated in the closed conformation. The DHF-H^+ and NADPH ligands are shown in the closed RS configuration.

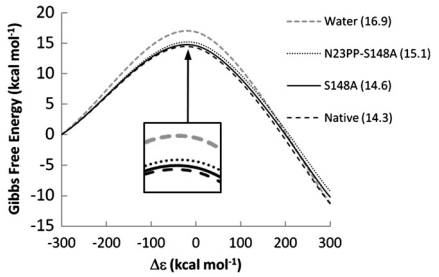

Fig. 2. Average EVB free-energy profiles for the reference reaction in solution (long gray dashes), WT EcDHFR (long black dashes), the N23PP-S148A mutant (short black dashes), and the S148A mutant (solid black line). The numbers in the brackets denote the corresponding activation barriers, in kilocalories per mole.

counts for the difference in the activation barriers. We may continue the analysis and ask whether the change can be classified in terms of either of the models presented in Fig. S1, which describes the correlation between $\Delta g^{\neq}$, λ (or λ'), and ΔG_0. However, here we are dealing with an extremely small change in the catalytic effect (in clear contrast to the large and interesting case addressed in ref. 8), and with a complication due to the significant effect of the change in the donor and acceptor distance (which makes it difficult to distinguish between λ' and the effect of the work function). Thus, our main point is that ΔG_0 has changed due to the mutation, reflecting changes in both the work function (i.e., bringing the donor and acceptor to a closer distance), as well as λ', which in turn leads to the changes in the activation barrier. Now, instead of trying to quantify the small change in λ', which is less stable than $\Delta g^{\neq}$, we only have to establish that the electrostatic effects change upon mutations. We have established this point by providing the linear response approximation (LRA) estimate of the contribution from each of the protein residues, in the WT and mutant enzymes, to the total free energy of charging the corresponding RS (Fig. S4). The finding that the preorganization of the native and mutant forms of DHFR are in fact different from each other is a complete contradiction to the assertion of ref. 6 (which was based on only examining structures, the perils of which we already touched on in the section titled *Background*). Additionally, the differences in contributions shown in Fig. S4 of course cannot be evaluated by simply visually examining the structures, without computational tools.

Flexibility and Conformational Dynamics. Having proven that the mutation is actually changing the reorganization energy, and that this accounts for the corresponding change in the catalytic proficiency, we may still explore the effect of flexibility and dynamics. That is, ref. 6 established that the N23PP/S148A mutant cannot access the occluded configuration, which is available to the product ternary complexes of the WT. Not being able to access the occluded conformation was in turn taken as a potential sign that the flexibility of the active-site residues may in turn be reduced (which NMR dispersion experiments showed to be the case), and this reduced flexibility was then presented as the reason for the (minutely) greater activity of the WT compared to the mutant enzymes.

Because ref. 6 has not provided a clear definition of the presumably catalytic conformational coordinate, we find it necessary to define this coordinate, and the most likely coordinate (that might be meant by the discussion in ref. 6) is the one going in the direction of the closed to occluded (CL → OC) transition (note that the authors also make the assertion that "the fact that the mutant enzyme is unable to adopt an occluded conformation suggested that the flexibility of the active site loops may be considerably dampened," further emphasizing that this is the only reasonable interpretation of their arguments). This coordinate is examined in the present work, but it should be emphasized that our analysis is general and not limited to this specific transition.

Based on this assumption, we evaluated the free-energy landscapes for the WT and mutant enzymes along the chemical and conformational coordinates. The dependence of the surface on the conformational coordinate was estimated by means of a specialized version of the LRA approach (see, e.g., refs. 16 and 17) and the results for the full (CL → OC) transition are given in Table S2. As seen from this table, it is clear that, in the case of the mutant, the occluded state is higher in energy in both the RS and PS, which accounts for the fact that the conformational transition from the closed to occluded state is not experimentally observed for the mutants presented in ref. 6. Next, we evaluated the complete catalytic landscape along the CL → OC path for the WT and mutant enzymes, and the corresponding results are shown in Fig. 3. As can be seen from this figure, the surface for the mutant is similar to the WT, but with a higher barrier and higher free-energy difference between the occluded and closed states. Furthermore, Fig. 3 demonstrates that we are able to reproduce the fact that the surface is shallower (and thus more flexible) along the CL → OC path in the WT RS. However, the difference in catalytic efficiency is completely accounted for by the "flexibility" in the shape of the surface along the reaction coordinate (rather than the conformational coordinate). It should be noted that the issue above was more apparent in our previous related study (15), which is discussed in more detail in the *SI Text*.

In order to highlight the fundamental problems with the dynamical proposal, we start by schematically illustrating our point in Fig. 4, which considers the nature of the reactive trajectories in the landscape of the conformational and chemical space. As seen from the figure, one may consider two types of trajectories: (*i*) trajectories that go directly from the CL state to the CL transition state (TS) (CL TS), and (*ii*) trajectories that only move in the direction of the OC state [as far as, say, a partially closed/occluded

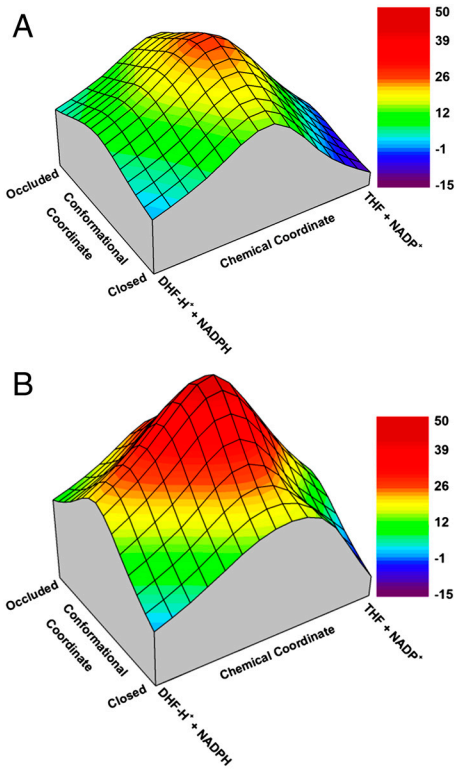

Fig. 3. Free-energy landscapes (in kilocalories per mole) for both (*A*) wild-type EcDHFR, as well as the (*B*) N23PP/S148A mutant. The energetics along the conformational coordinate was examined using a specialized version of the LRA approach, which allowed us to estimate the conformational energy for the transition between the closed and occluded conformations (see also, e.g., refs. 16 and 17). Note that the results for the full (CL → OC) transition are also provided in Table S2.

(CL/OC RS) structure] and then move back toward the CL TS. Following the assumption of ref. 6 that the impairment of the catalytic activity of the enzyme is not a simple effect of blocking the transition to the occluded structure, we do not explore motions to the occluded TS.

Now the first point to consider here is that, by any physically based analysis, we must conclude that the system obeys the equipartition theorem (if the barriers are relatively high), and therefore that the chances of being found at any specific point on the landscape are determined by the corresponding Boltzmann probability (see ref. 18). Thus, no dynamical sampling effect can change the final outcome. Secondly, the availability of the CL/OC region does not help accelerate the reaction, as the mean passage time for path 2 (CL RS → CL/OC RS → CL RS → CL TS) must necessarily be larger than that of the CL RS → CL TS transition (as there is no memory of the transitions and we would simply have to spend extra time in the path). In other words, there is basically no catalytic advantage to having a complex landscape with the system passing (however briefly) through the CL/OC state.

The analysis presented in Fig. 4 can be supported by either repeating the renormalized simulations presented in our earlier study of adenylate kinase (AdK) (18), or even by performing explicit simulations because the conformational changes in the present case are smaller than those in AdK. Here, we only present qualitative support, using explicit simulations (see *SI Text*). We also note that our earlier work (18), which presents a completely general model for any enzyme with a reasonable friction, demonstrated that there is no way by which the conformational barrier can be "remembered" in the chemical step once the chemical barrier is greater than a few kilocalories per mole (1 kcal = 4.18 kJ). Thus, we do not find it useful to repeat our previous calculations (beyond what we have included in the *SI Text*) because, for example, the study presented in figure 3 of ref. 18 provides an analogous scenario to what we would obtain by examining the CL → OC (or just the CL/OC → CL) path in the present case. Overall, the authors of ref. 6 were unable to provide any direct evidence that the millisecond conformational motions are helpful to catalysis, except for the argument that k_{hyd} changes when the occluded state is not accessible and the active-site residues lose their flexibility. However, our present work demonstrates that the effect of the mutation can be fully reproduced by the change in the activation barrier, in a model that also reproduces the experimental observation about the shape of the surface along the conformational and chemical directions. Now, because (*i*) an (undefined) dynamical coupling was only implicated and not actually observed in ref. 6, (*ii*) the relevant observables were reproducible by the simulations, and (*iii*) the validity of the authors' dynamical model would require a violation of the equilibration time dictated by the biological friction range (which would in turn violate everything that is known about vibrational relaxation in large molecules), we have to conclude that the dynamical model of ref. 6 is invalid.

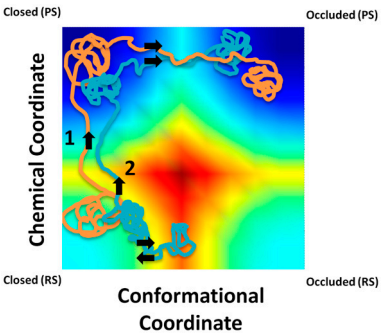

Fig. 4. An illustration of the relevant motions in the landscape of the conformational and chemical space. Shown here are two different possible paths, comprising (1) trajectories that move directly from the closed state to the closed TS, (2) trajectories that first move from the closed state in the direction toward the occluded state, and then move back to the closed state and the closed TS.

Another interesting issue is the idea that there exists dynamical correlation between the conformational and chemical motions. We explored this issue in relation to DHFR (15) by evaluating the normal mode vectors that represent the multidimensional reaction coordinate (which was calculated as the difference between the RS and PS), as well as the vector that represents the folding coordinate (or the motion to the occluded state) in the mesophilic form of *Escherichia coli* DHFR (ecDHFR). This study, which is summarized in the *SI Text* (figure 9 of ref. 15 and Fig. S5 of this work), shows that the two coordinates are basically orthogonal. As in our previous work (18), we must emphasize that our study is not about the exact "dynamics" in the conformational direction because our conclusions are general and applicable for any reasonable behavior of the system.

Entropic Contributions Are Free-Energy Contributions. There might be some confusion about the fact that the potential energy surface determines the free energy, including the entropic contributions, as well as the fact that flatter surfaces lead to higher entropies (more negative $-T\Delta S$), and also lower frequency motions. However, the motions are a *result of* and never the *reason for* the topology of the surface or the change in free energy. Now, in principle, having a flatter surface in the conformational direction in the product (and the TS) than in the RS can lead to a positive activation entropy and thus to a reduction in the barrier [see the discussion of the entropic effects in alcohol dehydrogenase (ADH) in ref. 19]. Here, one can benefit from observing an increase in flexibility in the product state of the WT relative to the mutant enzyme, and this can be helped by NMR studies such as those of, e.g., refs. 20 and 21. However, one must be able to specifically look at the difference in conformational flexibility (that is orthogonal to the reaction coordinate) and not at some general flexibility. More importantly, what counts is the actual flexibility in the orientation of the protein dipoles (as discussed in ref. 19 in the case of ADH), which is of course included in our free-energy calculations. Thus, it would be useful to see if we have a significant entropic contribution to catalysis (overall, as shown in ref. 22, the contribution is not large) and then to see how it correlates with NMR studies of the RS and PS (because we do not see a chance for relevant experimental information on the TS). Now, such studies will not be able to show that the motions lead to catalysis. However, having more large amplitude motions in the direction orthogonal to the reaction coordinate may reflect a situation (see the second case in the discussion of figure 10 of ref. 4) where the surface is less steep in the WT and may (or may not) lead to an entropic contribution to $\Delta g^{\neq}$.

Discussion

Recent years have seen an explosion in interest in the idea that there exists a connection between millisecond dynamics and enzyme catalysis (for a detailed review, see ref. 4). The popularity of this idea persists, despite extensive evidence to render it controversial (4, 18, 23). However, a recent work is noteworthy (6) in its assumption that the reduction in the rate constant of the presented DHFR mutants is because of the elimination of the dynamical coupling to the occluded conformation. For this deduction to be valid, one has to be able to dismiss the rather obvious idea that the mutation changes the activation energy by changing the electrostatic preorganization. Thus, it was argued that, because the native and mutant enzymes have "very similar" structures, they also have similar reorganization energies. However, our quantitative simulation studies have established that the electrostatic preorganization is actually quite different in the native and mutant enzymes, which also shows that simply inspecting protein structures cannot assess the preorganization. For example, just

looking at an NMR structure (in contrast to using it in careful calculations such as in ref. 9) cannot predict either electrostatic stabilization or pK_as (let alone the TS energy) because this requires the intervention of reliable computational tools.

More important, however, is the fact that we were able to reproduce the observed trend in the mutational effects by EVB calculations (without adjusting any parameters for this purpose), and thus have shown that all the observed changes in k_{hyd} are accounted for by the activation free energies. Therefore, we have established that the catalytic effect does not need to be explained by esoteric dynamical effects.

Next, we explored the significance of the differences between the conformational landscapes of the WT and N23PP/S148A mutant, and we showed that having access to motions in the direction of the occluded state does not help catalysis, either by dynamical coupling or by an increase in flexibility (short of perhaps small nondynamical entropic effects). We also clarified recent catalytic concepts and their interesting implications. For example, one of the concepts presented in ref. 6 is the idea of sampling high-energy conformations (e.g., refs. 24 and 25), which is formulated, for example, by stating "although the active site of the N23PP/S148A ecDHFR mutant is fully preorganized in the ground state, millisecond-time-scale fluctuations of the active site are restricted so that the enzyme cannot efficiently sample higher-energy conformational substates that are conducive to formation of the transition state." Unfortunately, there is a serious problem with this idea, as touched on in the previous sections. That is, it is true that NMR allows us to probe the motions that are associated with relatively high-energy regions. However, the observation(s) of motions on the millisecond timescale by NMR has little to do with the chance of reaching regions along the path to the TS. The sampling in large molecules is completely dictated by the potential energy surface (which leads to the free-energy landscape) and, once the barriers are greater than a few kilocalories per mole, the sampling follows the Boltzmann probability. Thus, the only way to reach the TS in a timescale of 1/20th of a second (16 kcal/mol) in the mutant is for the mutant to pass regions that correspond to millisecond fluctuations (15 kcal/mol regions). The considerations above mean that nothing can either increase or decrease the chances of visiting high-energy points on the free-energy surface (along the reaction coordinate), except changing the free energy of those points, because the probability of sampling them is completely determined by the exponential of the free energy (i.e., $\exp[-\Delta g(X)/RT]$, where X designates the reaction coordinate). This point has been proven in our simulation study of AdK (18) and there is no experimental finding that points to the possibility of inertial sampling in the case of millisecond barriers (note that the argument in the most recent experimental findings, ref. 6, was based on the assumption that the reorganization energy does not change, which we have shown to be incorrect). Apparently, the relevant issue here is not the sampling process itself, but rather the probability that different points along the reaction coordinate will be sampled. The sampling process is in fact rigorously defined and accurately simulated by our free-energy simulations, as is demonstrated in the *SI Text* (i.e., Fig. S6).

In considering the assertions of ref. 6, it is useful to address the concept of fluctuations that are needed to bring the active site to "a tightly closed form" and are blocked in the mutant. That is, the RS is at a free-energy minimum and there is no need for fluctuations to bring the system to this tightly closed active site (see figure 7 in ref. 4 and Fig. 4 in this paper). In contrast, if the authors mean that we have some sort of blockage of the fluctuations that lead to the TS, then we do not see any evidence for this either. Additionally, if the authors mean fluctuations on the TS region, then we have here some ill-defined relationship to activation entropy that is neither defined nor properly formulated. Finally, note that simply arguing in the reverse direction—i.e., that the fact that the mutant cannot adopt an occluded conformation might be an indicator that the flexibility of the active-site loops may be considerably dampened upon mutation—does not in and of itself define a proper flexibility coordinate, nor does it make it clear what the authors believe the purpose of this flexibility coordinate actually is.

It could be argued that our study has not addressed the dynamical proposal of ref. 6 because this study never explicitly identified the dynamical motion as motion toward the OC configuration. We must once again reemphasize that we expended great effort into investigating what was the proposal put forth in ref. 6 actually is (because this work was unclear about the observed dynamical effect). Therefore, we chose the most reasonable interpretation of ref. 6, which would be to examine conformational fluctuations in the direction that is being blocked by the key mutations.

The importance of the active-site preorganization can be clearly seen in the case of enzyme design. Here, despite significant effort in this direction, most attempts at rational enzyme design have met with limited success (see discussion in, e.g., ref. 26 and the *SI Text*). However, such concepts can be lost behind arguments that claim that the active site is still fully preorganized upon mutation—once again, the preorganization is not about the *structure* but rather about the *work* involved in rearranging the protein from its RS to its TS. In any case, the observed structural changes in the D-A distance (from 3.3 to 2.9 Å) are sufficient to render any structure-based assumption of identical electrostatic reorganization irrelevant. That is, in water, for instance, such a change would decrease the solvation energy of an ion pair by about 15 kcal/mol (see equation 7 in ref. 27 and related discussion therein). The fact that the distance in the mutant is smaller arises most likely as a result of an increase in orthogonal reorganization (i.e., an increase in solvation energy). It is also useful to note that enzyme design efforts will benefit far more from direct calculations of the reorganization energy than from looking for the link between conformational motions and the chemical step (a link which is frequently suggested by the proponents of the importance of dynamical contributions to catalysis).

Our work focused on the recent attempts to relate mutational studies in DHFR to the dynamical proposal (6). However, it is worth pointing out that the same type of attempt has emerged in another high-profile study of cyclophilin A (7). Here, once again, the finding that mutations that block interconversion between conformationally different substates also reduce catalytic activity was used as proof for the existence of some sort of dynamical effect. However, as we discussed in our recent study (4), this specific system does not provide a causal and logical connection between the interesting and important experimental observations and the presumed dynamical effects. Here, again, it is obvious that active sites with different preorganization (because of a different sequence) will have different activation barriers and, at present, there is nothing in the reported experimental study that would allow one to determine the preorganization without computational modeling (which, if done properly, would certainly give different results for the two systems and will, based on any other previous case, reproduce the observed mutational effect without being dependent on the interconversion between substates).

It is also important to address the assertion that the motion of the Met20 loop (6) is related to the motions of the protein in the TS and to catalysis. We note that we have no problem finding all the modes of the protein that have projections on to the reaction coordinate, have been doing so for many years (19, 28), and have already done so for this system (see *SI Text*). However, the effect of moving in the OC direction is described in Fig. 3 more carefully than would be possible by just looking at the normal modes of the system. Now, upon examining Fig. 3, we do not find a major advantage for moving in the conformational direction (see the TS ridge). Moreover, even if there were a significant effect from movement in the occluded direction on the TS (i.e., the TS would be shifted in the occluded direction), this would have little to do

with dynamics. That is, as we have shown since 1984, the coordinates of the enzyme [referred to in our works as generalized solvent coordinate (e.g., figure 6 of ref. 19, and ref. 28)] and not just the substrate are part of the overall reaction, in the same way that the water reorientation is a part of the reaction coordinate in water. Thus, moving along the enzyme coordinate is a part of the free-energy landscape, and it does not explain any catalysis (catalysis is explained by the factors that *change* the surface and not by the resulting surface itself).

Interestingly, the possibility of having a shallow TS along the conformational coordinate of the WT means that the overall $-T\Delta S^{\neq}$ is reduced. The same will be true for the product entropy, *if* the conformational coordinate is shallower in the product state. Of course, these entropic contributions are evaluated in our free-energy calculations, but the fact that NMR studies can help in exploring entropic effects (29) is important. It would of course be interesting if the activation entropy were found to be more positive in the WT than in the mutant, but it is crucial to clarify that the entropy is a well-defined free-energy effect that has nothing to do with dynamics (in fact, the potential motions along the TS ridge will be in the nanosecond and not in the millisecond range). At any rate, experimental correlation between NMR and the activation entropy requires at least separate information from the RS and PS.

Here we have addressed the main points raised in the recent relevant experimental studies. However, because the issues being explored in this work are controversial, it is not possible for us to address every claim or finding of the proponents of the dynamical proposal before we are even aware of it. Nevertheless, we attempt to provide a useful clarification of this issue in the *SI Text*, including a discussion of general problems in addressing catalysis (see, e.g., Fig S7). Overall, we have herein provided clear evidence that so-called dynamical knockout mutations simply change the chemical step in catalysis by changing the activation free energy and *not* by any dynamical effect. Further experimental challenges of our findings will have to come with detailed and consistent computational interpretations, or with direct demonstrations of a dynamical coupling (such as, e.g., probing the correlation between the conformational and chemical motions), rather than circumstantial arguments based on indirect proofs.

Methodology

All EVB calculations were performed using the MOLARIS simulation package and the ENZYMIX force field (30). The EVB activation barriers were calculated using the same free-energy perturbation umbrella sampling approach, which has been described in detail elsewhere (11, 31). The atomic coordinates that were used as a starting point in the simulations were taken from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (32) entries with access codes 1RX2 and 1RX4 for the CL and OC forms of WT DHFR, respectively, and 3QL0 for the closed N23PP/S148A mutant form of DHFR. The simulation systems were solvated using the surface constrained all atom solvent model (SCAAS) (30), using a water sphere with a radius of 20 Å centered on the substrate and surrounded first by a 2-Å grid of Langevin dipoles, and then by a bulk solvent. Long-range electrostatic effects were treated by the local reaction field approach (33). The free-energy perturbation mapping was performed in 25 frames of 10-ps length each for the movement along the reaction coordinate, using the SCAAS model, after the respective system underwent a 100-ps relaxation run. All simulations were performed at 300 K using a 1-fs time step. In order to obtain reliable sampling, the simulations were repeated at least five times with different initial conditions (obtained from arbitrary points in the relaxation trajectory after the initial 100-ps relaxation run) for each reacting system. The contributions of the different residues to the activation barrier were calculated by use of the LRA. Finally, the relevant ionization states were evaluated using the approach of ref. 30.

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