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REGULAR ARTICLE

Prechemistry barriers and checkpoints do not contribute to fidelity and catalysis as long as they are not rate limiting

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Abstract In the preceding article, "Perspective: Prechemistry conformational changes in DNA polymerase mechanisms" contributed by Schlick and coworkers as well as previous studies of these workers (Schlick et al. in Theor Chem Acc 131:1287, 2012; Radhakrishnan and Schlick in J Am Chem Soc 127:13245–13252, 2005; Radhakrishnan and Schlick in Biochem Biophys Res Commun 350:521–529, 2006; Radhakrishnan et al. in Biochemistry 45:15142–15156, 2006; Radhakrishnan and Schlick in Proc Natl Acad Sci USA 101:5970–5975, 2004) have argued that the conformational changes preceding the chemical step contribute to DNA synthesis and to the fidelity of DNA polymerases. In one of our previous investigations (Ram Prasad and Warshel in Proteins 79:2900–2919, 2011), we argued and showed that *as long as the free energy barriers associated with any of the prechemistry steps are not rate limiting*, they could not contribute to the catalysis and then to the fidelity. Though all our arguments are based on exact and well-defined scientific logics, Schlick and coworkers seem to overlook some of the clear conditions in these arguments and in particular the requirement that the chemical step is rate limiting in their arguments that the prechemistry barriers contribute to the catalysis. In fact, as long as the prechemistry steps are not rate limiting, we have shown that the enzymes cannot carry the memory of the previous steps. We also address other potential misunderstandings about several key issues; First, we clarify that it is misleading to relate the prechemistry proposal to the clear fact that the substrate-induced conformational changes determine the final preorganization (the issue is the height of the barrier of the enzyme substrate system and not the trivial fact that the enzyme has to change its structure when the substrate binds). Second, we address the presumed role of dynamical effects in enzyme catalysis and the assumption that any observable should be explored in studies of biological function even if they are not relevant to the given effect. Third, we clarify that the fidelity cannot be explained or quantified by invoking the induced fit or conformational selection effects but by evaluating the free energy contributions to the rate-limiting steps from the structures of the corresponding systems (that of course can reflect the induce fit structural changes). Overall, we put a major emphasis on clarifying what is the prechemistry proposal and thus on trying to force the reader to focus on the only real controversy. We of course dismiss any implication that our studies cannot explore mutational effects as we actually pioneered such computational studies and we clarify that in studies of chemical rates, the focus

Editor's Note: This paper and papers by Mulholland AJ, Roitberg AE, Tuñón I (doi:10.1007/s00214-012-1286-8) and Schlick T, Arora K, Beard WA, Wilson SH (doi:10.1007/s00214-012-1287-7) document and discuss contrasting outlooks on the questions of prechemistry and catalysis in DNA polymerase. All authors were initially provided with one another's manuscripts, at which point opportunities to make revisions were offered, and finally, Mulholland, Roitberg, and Tuñón were given the 'last word' on the revised manuscripts in their role as commentators. The editors of TCA hope that this discussion will illuminate key issues affecting ongoing work in this area.

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must be placed on evaluating the chemical barriers, rather than on irrelevant factors, but that the calculations of the chemical barriers must consider all the factors that determine this barrier (including metal ions) and also examine if needed different problematic proposals such as dynamical effects, tunneling, and prechemistry.

Keywords DNA polymerase beta · Prechemistry · Checkpoints · Enzyme catalysis

1 Background about the fundamental difference of views

In the preceding contribution [1] and in previous works [2–5], Schlick and coworkers (SABW) have argued that prechemistry is important for the replication fidelity of DNA polymerase β (Pol β). Their present clarifications on this subject are a response to our earlier conclusions [6] that the role of prechemistry in determining the fidelity is minimal and are placed in the framework of discussing our more general findings [7] about the unimportance of dynamical effects in enzyme catalysis.

We start with the analysis of the clear and well-defined argument between SABW and us: they believe that prechemistry steps are important for the replication fidelity even if neither of these steps is rate limiting, while we assert that this is incorrect. To clarify this crucial point, we must go in two directions. First, we present what we believe to be the essence of the opposing views in Fig. 1. This is done since we believe that looking at this figure is by far more effective than reading the exact quotations, which tend to change with time, and frequently are not well defined (see below). At any rate, the figure considers two cases—one where the prechemistry barrier is lower than the chemical barrier (Fig. 1a), and the other when the prechemistry barrier is higher (Fig. 1b). We state that in the first case (and only in the first case), the prechemistry barrier does not affect fidelity, whereas SABW propose that in the first case, the prechemistry barriers contribute to fidelity. Now to establish that this is what is being stated, we bring first PW statement (Ref. [6]) "*As long as the free energy barriers associated with any of the prechemistry steps are not the rate limiting they could not contribute to the catalysis and then to the fidelity*" and contrast it with SABW current statement "*Thus, substrate-induced conformational changes that assemble the polymerase active site prior to chemistry contribute to DNA synthesis even when they do not represent actual rate-determining steps for chemistry"* or their previous statements (e.g., "*The conformational rearrangements and cooperative motions discussed here likely represent key mechanisms used by polymerases to enhance fidelity [4])"*. To prevent possible confusion, we must clarify that the part in SABW statement on whether it is or it is not on substrate-induced changes, has nothing to do with the controversy; Of course, it was always about what happened to the free energy surface when the polymerase binds to the substrate as this is what determines the final chemical barrier and fidelity. Thus, the profile drawn in Fig. 1 is of course the one with the effect of the substrate and all other factors included in configurational averages that determine free energies. The fact that any discussion is about the behavior of the entire system is also crystal clear from all the barriers drawn in previous papers of Schlick and coworkers (e.g., Refs. [2, 4, 5]) and of course those evaluated and considered by Warshel and coworkers [6, 8]. Thus, it all boils down to the validity of the above PW statement and to earlier statements from our group. In fact, the whole idea of checkpoints [4, 9] is clearly about the effect of the details of the conformational landscape before the chemical step on the overall rate. Here, PW asserts that despite the intriguing nature of these barriers they cannot contribute to fidelity if they belong to case A in Fig. 1. We also like to note that the confusion already starts with labeling of the open and closed ternary complexes (Fig. 1). That is, for at least 35 years, we and others have used the symbol ES for the fully folded and preorinted state of the protein–substrate complex and not some point on the path to this state (we retain this notation in this paper (Fig. 1)). Considering incorrect state for ES compounds, (ES' in SABW work), lead to the incorrect claim (see below) about the role of conformational changes in determining the preorganization of the chemical barrier. Confusing the barriers along the conformational path with the fact that we have structural changes is simply misleading. In fact, Warshel was the one who quantified and demonstrated the role of folding on catalysis [10]. We hope that the reader will keep these issues in mind while reading the exchange. Below, we will also discuss other assertions made by SABW and clarify what are the factors that are relevant to replication fidelity.

2 Clarifying what are the actual issues and how to judge the validity of different views

As we already eluded to above, any discussion of the arguments on the prechemistry controversy is futile without a clear and unique definition of the relevant disagreements. Thus, we expand and clarify below what are the actual assertions of SABW and how to explore their validity without falling into semantic traps.

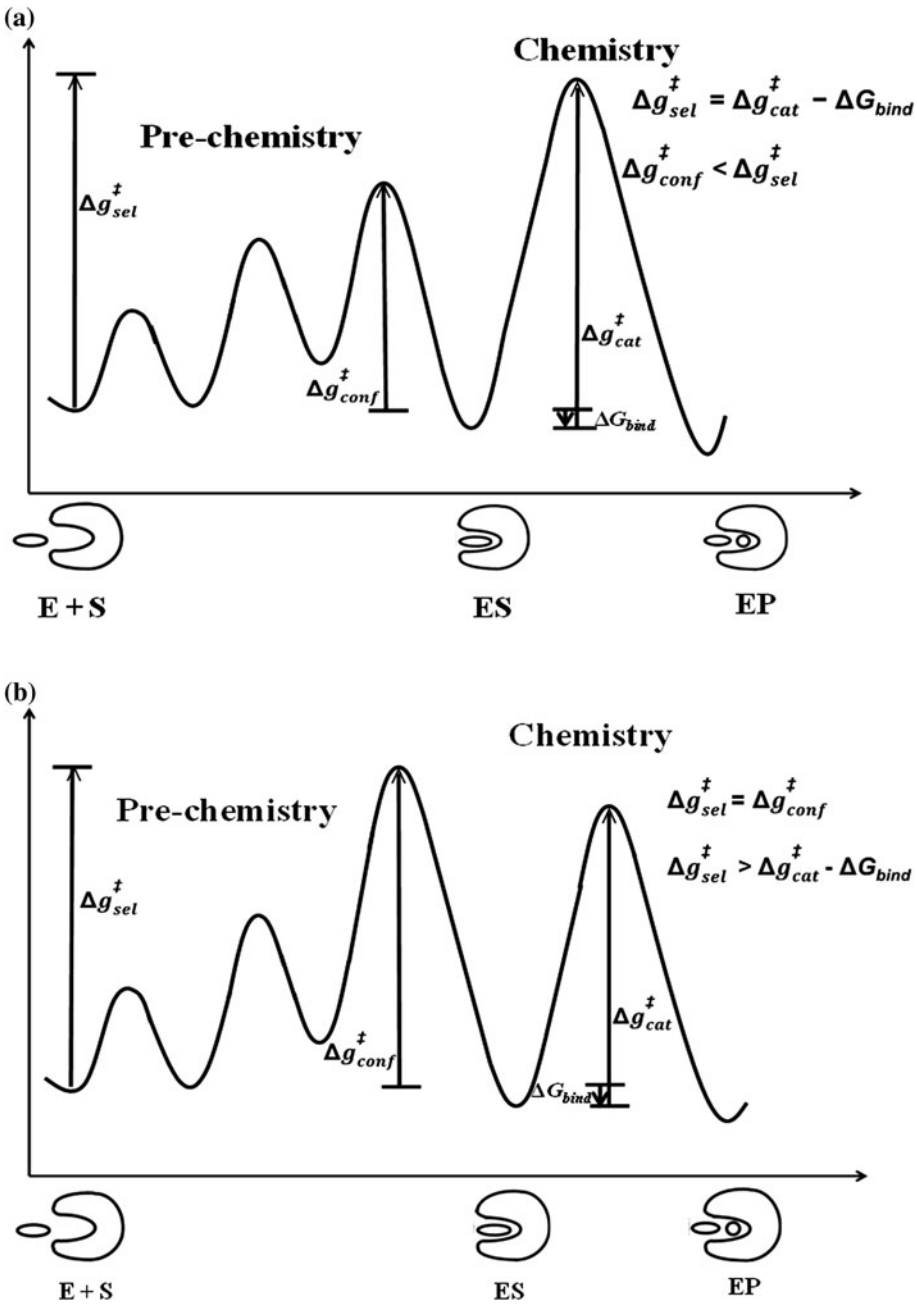

Fig. 1 Illustrating the exact meaning of the prechemistry proposal. Case (**a**) corresponds to the situation when the chemical barrier is higher than the prechemistry barrier (in this case, the chemistry step is rate determining). Case (**b**) corresponds to the situation when the prechemistry step is the rate determining. SABW argue that even in cases like **a**, the prechemistry events of the landscape would determine the overall rate. On the other hand, we maintain that the lower barriers associated with the prechemistry events as in case (**a**) will have no effect on the overall rate. In other words, we maintain that the highest activation barrier (relative to the unbound state) absolutely determines the overall rate and that anything that happened during the passage through the lower barriers and checkpoints will be forgotten in the passage of the highest barrier. Of course, when the prechemistry barrier is higher than the chemical barrier (case **b**), it will determine the overall rate, but this is completely consistent with our proposal, and should not be confused with the presumption that we suggested that when the prechemistry is rate determining it *will not* determine the overall rate. We also like to clarify that we label the state before the chemical process as ES (and not as ES* as SABW do or ES' as MRT do)

2.1 Prechemistry and catalysis and what has actually been proposed by each side

In considering the main issue (namely, the checkpoint prechemistry proposal), it is really not useful for the reader to assume that the opinions that are brought without quotations actually reflect what is the original view. Thus, we would like to point out again that the argument about the validity of our (or other) scientific work must address what was actually said and found, and not what the authors *think* was said and found. A crucial illustration of this would be the statement in Ref. [1] that "*Warshel and Prasad argue that conformational adjustments preceding the pol β chemical step are inconsequential to understanding catalysis or fidelity*". This is simply a misleading assertion, as can be judged based on the above PW quotation about the identity of the rate-limiting step, or by considering the discussion defining the controversy and the points below. In fact, we never made any statement about the role of conformational changes or folding in connection with the prechemistry controversy, and very clearly talked on the conformational *barrier* leading to the state before the chemical step. The barrier associated with the conformational adjustments is indeed inconsequential to the magnitude of the catalytic effect if it is not rate limiting, but of course the

conformational changes by themselves contribute to the chemical barrier. In fact, Warshel and coworkers have pioneered studies on the relationship between the conformational landscape and the chemical barrier.

After clarifying this mischaracterization of what was stated in all of our relevant papers,we hope to show here is that our arguments and findings are, in fact, very solid and cannot be negated by any of the arguments presented by SABW (or any other current work we are aware of).

Since the above assertion that PW argued that conformational adjustments preceding the chemical step are inconsequential to understanding catalysis or fidelity may still sound convincing to those who have not invested significant time into studying the relevant studies, we briefly clarify what was actually found. That is, what has actually been argued and established very clearly by us [6], is that the rate of the chemical step is *independent* of the activation *barriers* of previous steps, as long as the chemical step is rate limiting. Therefore, understanding catalysis and fidelity has little to do with understanding the conformational changes before the chemical step, *as long as* we know the actual structure of the preorganized active site, and as long as the chemical step is rate limiting.

When SABW finally get in their discussion to the main issue, which is the validity of the prechemistry proposal in DNA polymerase β (pol β), this proposal does not seem to be presented in a clear and scientific way. Here, we must resort to the rule emphasized in [7], where, in any argument on a definition, one *must* use the definition given in the original presentation of the controversial concept. In fact, the clearest and most logically valid argument for the prechemistry concept has been given in [11, 12], where it was argued that actual calculations produced a major barrier before the chemical step, and this barrier, which is apparently smaller than the chemical barrier, plays a major role in determining the fidelity. Incidentally, our simulations indicate [6] that the prechemistry barrier calculated by Schlick and coworkers is an artifact of fixing the enzyme in the minimization process [11, 12] that calculated the barrier. More importantly, the prechemistry barrier that is lower than the chemical barrier was still claimed by SABW to contribute to pol β replication fidelity (see Sect. 1 for the exact definitions and quotations). However, as we very clearly demonstrated in our earlier work [13], a low conformational barrier cannot contribute to the actual overall rate. Our computational proof cannot be contradicted by any circular argument, or indirect experiment (no direct experiment that can consistently show that the inertial effect of the conformational motion can be used in the chemical step exists). In fact, only simulations can be used to contradict our simulation results but no such work has been presented.

As further support for the prechemistry idea, we are also told about the existence of conformational changes that lead from the unfolded to the correctly folded structure [1] (which has to be correctly preorganized for catalysis). However, we have never challenged the existence of such conformational changes (and in fact, we have studied the corresponding barriers, see e.g., [6]). Unfortunately, the time and path of this change is irrelevant to the final active site structure and thus to the chemical barrier and the rate of passing this barrier.

2.2 The meaning of catalysis and fidelity

While SABW paper raises some interesting issues, it unfortunately ends up with major misunderstandings about enzyme catalysis and fidelity. Part of the problem is associated with the fact that many of the quoted discussions and analysis of proposals on the origin of catalytic (or other) complex biophysical effects are not useful, because they are not clearly defined and frequently misleading [7]. Thus, in order to further clarify these issues, we reiterate [6] that as long as the barrier of the chemical step is higher than all other previous barriers (as is the case in Pol β [14]), then the catalytic turnover of the enzyme (k_{cat}/K_M) as well as the fidelity is determined by the chemical barrier and substrate binding equilibrium. These enzyme properties are *not* affected by the lower barrier of any "prechemistry" step (which have been named prechemistry checkpoints [4, 9]).

2.3 The role of enzyme dynamics

SABW start by mentioning the dynamical proposal (which they appear to be in clear support of) [15, 16], with a level of bias that, in our opinion, does a disservice to the field. That is, it is argued that "*it has also been generally concluded in the above cited reviews that reliable molecular simulations have an important role in elucidating structural changes during enzyme catalytic cycles, including achieving reactive conformations, even if they do not support a catalytic role for dynamics in driving the chemical reaction per se*". Unfortunately, and as is clearly documented in Ref. [7], many works have incorrectly concluded that dynamical contributions are important for catalysis. Furthermore, bringing up Karplus' paper [16] without mentioning our response that was published in the same journal issue [17] presents a distorted picture of the field.

The dynamical proposal, with a clear implication that it is crucial for catalysis, has been extremely popular since the 80 s [18–22]. Only few works have challenged validity of this proposal by presenting careful computational analysis of any possible aspects of this proposal [7, 13, 17, 23–26] (including at present the only valid study of the dynamical role of the coupling between conformation changes and dynamics in the long time accessible to

experiment studies [13]). Other theoretical studies that gradually started to support the idea that dynamics does not play significant role in catalysis have only started to appear recently [27, 28]. In parallel, one proponent of the dynamical proposal started to tune down and to modify their proposal (e.g. see our response to Ref. [16] in [17]). We raise this point since the only way to resolve the validity of the dynamical proposal is to carefully define what has actually been stated by other workers and then to discuss what has actually been found when these statements have been carefully analyzed.

SABW also bring additional issues such as induced fit and conformational selection to support their view. Here, we will show that either the implication that we disagree with some assertions is incorrect or that the corresponding discussion has very little to do with the controversies are examined here.

2.4 Catalysis, catalytic cycles, and the role of dynamical effects in controlling catalysis

SABW argue about the existence of accumulated evidence that prechemistry steps are crucial, and that the chemistry is just one part of the picture needed to understand *the complete catalytic cycle*. Of course, approaches that just look at the prechemistry can never account for any single observed rate constant except in the obvious case, where the prechemistry step is rate limiting (which is irrelevant to our present discussion). But even their argument about the need to gather pre-chemistry details to understand the complete catalytic cycle, although sounding deep, just reflects the far-too common confusion between the overall catalytic cycle and actual catalysis, that keeps being propagated by some researchers in the field (e.g., [29]). For example, it has been argued [29] that the enzyme's catalytic cycle can be compared to "*the performance of dancers with basic common steps as foundation and open-ended sophistication and creativity needed for individual style and execution. Moreover, the choreography ultimately creates the flow of the entire dance*" (or, more to the point, that each step in the catalytic cycle is tightly linked, driving the subsequent step). Similarly, it was argued in [29] that "*By definition enzyme catalysis has to be a multi-step journey due to binding, chemistry and release*". This is clearly an elegantly phrased proposition, but it somewhat misses the point. First of all, for the idea about the need to follow the entire "dance" to in any way be the case, the enzyme will have to actually have a memory of previous steps, which, as we have demonstrated in [13], is not the case. The second weakness of this argument [29] stems from an apparent misunderstanding of the concept of catalysis, which can be seen in Fig. 1 of [29] where the authors present their view of a free energy landscape for enzyme catalysis. The problem is that in this figure, the authors have mistakenly taken a factor of $\sim$2 difference between the rate constant for the chemistry and for product release (which is usually slower in the enzymes than the chemical step by a factor of $\sim$2–100) and drawn this as a factor of $\sim$2 in the *free energy*. This is hugely problematic, because a difference of $\sim$100 in rate is almost negligible in terms of free energy, and, anyhow, the issue is not a small difference of $\sim$100 in rate constant, but rather the several orders of magnitude rate acceleration relative to the corresponding uncatalyzed reaction in solution. A corrected version of the energy landscape for enzyme catalysis (based on the incorrect landscape drawn in [29]) is presented in Fig. 2a. Specifically, this figure illustrates the different steps in the catalytic cycle, highlighting (in its caption) the main points. In particular, the figure clarifies that once the enzyme has achieved such a huge reduction in free energy, it is then possible that a different step in the catalytic cycle will become rate limiting, but, this is clearly not the origin of the catalysis.

Going back to the actual arguments presented by SABW, we must clarify that, as long as the chemical step is rate limiting, there is no *single* valid piece of evidence that any other factors (except the activation free energy of the chemical step) can determine the catalytic effect. Of course, one can mention countless references that presumably suggest the opposite, but none of them even come close to providing solid support for their arguments. Here, we emphasize once again that the only way forward is to carefully analyze specific arguments, as has been done in Ref. [7].

2.5 Induced fit and conformational selections are irrelevant to the prechemistry argument

SABW put a significant emphasis on the induced fit concept in trying to support the prechemistry idea. However, as discussed in great length in Refs. [6, 8], the induced fit idea (which is an interesting observation) does not explain *any* catalytic or fidelity effect nor can reproduce it (despite constant attempts to invoke a relationship between the two). Basically, Koshland's induced fit proposal [30] only tells us that ligand binding changes the structure of the active site, but it does not tell us what the new active site is, and never addresses the issue of what it takes to obtain better catalytic activity (namely it never came even close to addressing or proposing Warshel's preorganization concept [10]).

Invoking the influential induced fit proposal among the justifications for the prechemistry concept poses a serious problem. As a start, we are told that the enzyme was originally thought to be rigid, but, presumably, simulations show that there are active site motions. The fact is that

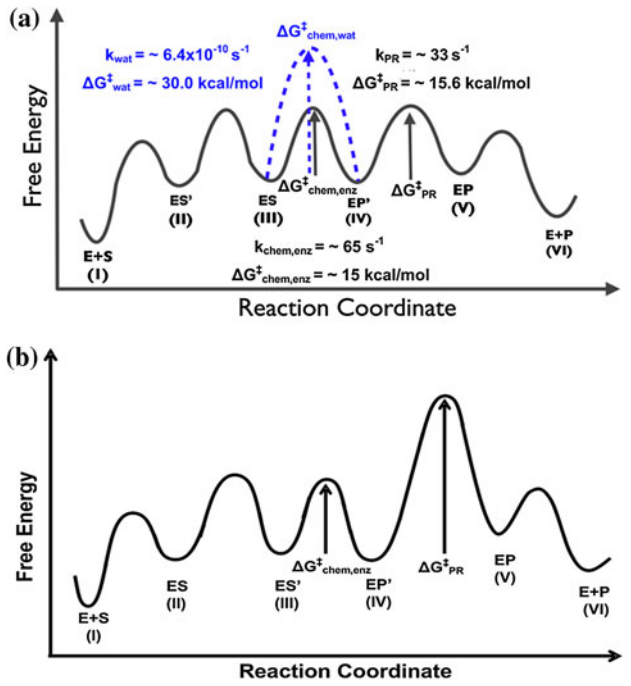

Fig. 2 A schematic representation of the free energy landscape of an enzymatic reaction, corrected from [29]. Shown here are the chemical steps in the enzyme and in solution (3↔4, designated as chem,enz and chem,wat, respectively), substrate binding (1↔2), potential conformational changes before and after the chemical step (2↔3 and 4↔5) and, finally, product release (PR, 5↔6). Note that, even for a chemical step on the millisecond timescale, this corresponds to a barrier of ~15 kcal/mol. The rate for product release can be slower than that of the chemical step, but this is usually by only a factor of ~2–100, which results in an almost negligible difference in the barrier. However, the difference between the barrier to the chemical step in the enzyme and in the corresponding uncatalyzed reaction in solution (*blue*) can be tremendous (in this particular example the reaction in water has an activation free energy of ~30 kcal/mol compared to 15 in the enzyme, but for a highly proficient enzyme this difference can be even greater). Once the enzyme has been evolutionarily optimized to lower this barrier, adjusting the relevant barrier heights to the other steps becomes a trivial issue and can even be necessary to achieve tight regulation of the enzymatic activity. This figure is intended to illustrate the traps of attempting to rationalize the tremendous catalytic power of enzymes without a proper energy-based analysis. Scheme (**a**) depicts the realistic trend in enzyme catalysis, whereas scheme (**b**) depicts the energetics conceived by [29], with an entirely unrealistic barrier for product release (no reaction will ever occur in a reasonable time with such a barrier)

starting already in 1976, [31] we never considered the active site to be rigid and have consistently explored all the relevant motions and relaxations. In fact, the problem with the studies of SABW is that in evaluating catalytic effects, they have not performed sufficient relaxation (see the establishment of this point in [6]). Second, the misunderstanding seems to be associated with the fact that SABW are talking about motion from the open structure to the closed structure of the complex, and these motions are actually irrelevant to the time of passing the chemical barrier or to the correct preorganization. In other words, as also demonstrated in Fig. 7 of [7] and Fig. 4 of [26], the preorganization of the Michaelis complex is *not* determined by path to this state.

SABW arguments about conformational changes (rather than conformational barriers) are further packaged with new fashionable terms such as "conformational selection" (for the allosteric effects), which may intrigue some readers. Here, we arguably have a major experience in actually modeling the real consequences of such effects in many systems [26, 32–35], and clearly in modeling the allosteric effect in DNA polymerases [6, 8] (Fig. 3). Therefore, we would like to point out that the allosteric effects are simply determined by the *balance* between the substrate binding free energy (or the interaction with other relevant effectors) and the free energy needed for optimal preorganization. However, this free energy balance has little to do with the prechemistry arguments brought up in [1]. That is, the conformational changes associated with the induced fit are simply changing the active site preorganization (see Fig. 3) and therefore changing the chemical barrier and the rate of the chemical step. Furthermore, the induced fit concept neither explains nor predicts the fidelity (see the discussion in [6, 8]).

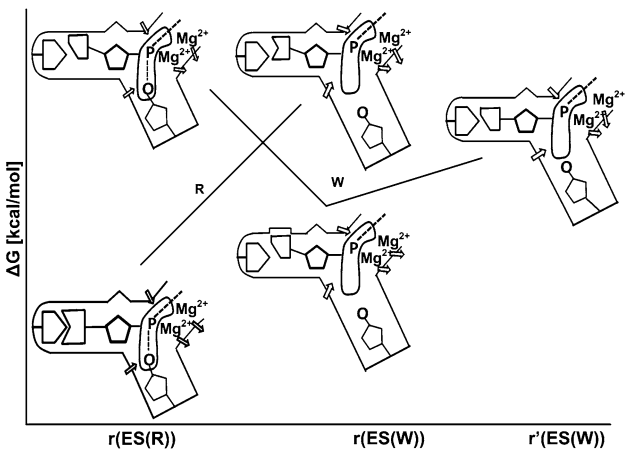

Fig. 3 Illustrating the origin of the allosteric effect. The figure considers the protein rearrangement upon binding of *R* and *W* at different protein configurations. r'(ES(W)) is the structure of the W before the relaxation at the base site. For simplicity, we did not consider r(TS) but the corresponding r(ES). In the case of *R*, the protein should provide optimal sites for both the chemical TS and base-pairing parts. On the other hand, in the case of *W*, the protein has to relax in the base-pairing site (to provide good binding) and this relaxation destroys the preorganization in the chemical part. The diagrams that represent the different configurations illustrate this allosteric effect where the arrows indicate the protein dipoles. In the *R* state, these dipoles are reorganized in an optimal way and thus provide maximum TS stabilization. On the other hand, in the W state, the dipoles are forced to assume less effective preorganizaion and thus yield less TS stabilization

It is important to clarify that even if one talks on "conformational selections" instead of induced fit we do not have a new insight about fidelity, since both terms represent basically the same effect (namely change of the landscape due to the presence of the substrate), where the difference in the pathway for the formation of the complex is simply a reflection of the shape of the combined free energy surface. In fact, as much as we are concerned the preoccupation with the difference between the two terms reflects in many cases an avoidance of the key issue, which is the calculation of the actual landscape. At any rate, talking on induced fit or on conformational selection is not going to change the fact that when the height of the barriers along the paths to our ES state is lower than the chemical barrier we will have no effect on fidelity. What counts here is the free energy of the final points and not the barriers (as long as they are not rate limiting).

2.6 Landscape effects

The elegant landscape described in Fig. 1 of SABW seems to be an attempt to convince the reader about the importance of the prechemistry and to connect this to the fact that the substrate binding changes the landscape (which is an induced fit effect). Unfortunately, such a landscape change is quite irrelevant to the prechemistry argument and also to the fidelity issues, *if the chemical step is rate limiting*. That is, we never had any problems with modeling the effect of the binding of the substrate on the conformational free energy and modeled such effects quite early [34–36], including in the case of Pol β [6, 8], using sophisticated and reliable ways of obtaining the complete surface, including the change in the chemical barrier along the conformational coordinate which was not quantified by other approaches. The problem with the implications of SABW is that they ignore the issue of the relative height of the chemical and conformational barriers. Here, if the chemical barrier is significantly higher than the conformational barrier, then we maintain that the prechemistry barriers have no effect. If the prechemistry barriers are higher, then they are rate limiting and completely irrelevant to the presumed effect of low-energy checkpoints.

The repeated discussion of the conformational changes, which were never questioned by us, is confused (as has been the case in many of the dynamical proposals, see e.g., [7]) as being "proof" of the dynamical idea in Pol β. That is, it is stated [2] that "*The studies are applicable to many other enzymes and further underscore the range and relevance of enzyme dynamics for understanding their complete mechanism*". Unfortunately, although enzyme dynamics studies (which were pioneered by one of us [37]) are interesting, they provide no help in understanding the fidelity, which is completely determined by the difference between free energy landscapes for the extension of the primer DNA strand by the correct and incorrect nucleotide. We would happily challenge SABW to try to reproduce any observed rate for this extension based solely on dynamical factors.

To gain further insights into the role of dynamical contributions to catalysis, we suggest the reader to compare reasoning of the Ref. [19], which presumably proved that dynamics is important in dihydrofolate reductase (DHFR), with our analysis of the same subject [7].

Here, it is worth pointing out that SABW invoke ref [19] without commenting on either the theoretical work [26] that demonstrated that the conclusions of [19] simply represent incorrect interpretation, or the subsequent experimental work [38] that further supported our findings (see also [28]). Arguments should be presented directly so they can be properly analyzed, or alternately, both sides of the argument should be presented.

It is also useful to point out here that the argument of Ref. [1] about the need to combine the complete landscape (and presumably the associated dynamics) in enzyme design applications is one of the best illustrations of the aforementioned problem in invoking dynamical contributions to catalysis. That is, in fact, to the best of our knowledge, the only studies that correctly considered the catalytic landscape (conformational and chemical space) in enzyme design efforts are those of Warshel and coworkers [39].

2.7 What are the experimental evidences for the prechemistry proposal?

SABW attempt to provide experimental proof for their argument by introducing the effect of the Arg283 mutation on fidelity as a proof of the prechemistry concept. This was done by arguing that "*Arg283 of wild-type Pol β interacts with the template strand in the closed substrate bound enzyme, but does not interact with DNA in the absence of a nucleotide. Replacing this arginine side chain through alanine substitution would be expected to destabilize the closed form of the ternary complex. The kinetic result is a loss of correct, but not incorrect, insertion efficiency leading to a dramatically lower fidelity. Thus, Arg283 plays an essential role in pre-chemistry events for correct, but not incorrect, nucleotide insertion*". Unfortunately, this assertion has a similar problem to that encountered in many of the dynamical proposals (e.g., [19]), in that an assumed relationship is confused with the actually observed one. In order to prove that Arg283 has anything to do with prechemistry effects, it is absolutely essential to demonstrate that the effect leads to some large prechemistry barrier (relative to the chemistry barrier). It is also crucial to show that the effect of the arginine is not the much more likely

effect of changing the activation barrier of the chemical step or changing the dNTP binding energy (i.e. free energy of the ES state (Fig. 1)). Now we are not aware of any single calculation that has correctly established the effect of this residue through a prechemistry effect, but, much more importantly (and exactly as in the recent case of DHFR [19, 26]), we have well-defined calculations that consistently account for the effect of the arginine mutations, through electrostatic transition state stabilization. That is, following the approach of Ref. [40], we calculated the electrostatic contribution of transition state binding free energies of the wild type and R283A mutants for both right (dCTP.G) and wrong (dATP.G) dNTP substrates. Our recent calculations of the TS binding energies (Table 1) reproduced the experimentally observed trend, where the mutation of R283A destabilizes the chemical TS of both right (R) and wrong (W) dNTP. In fact, similar calculated trend has already been reported in our earlier study [41].

A similar example is the author's consideration of the effect of the E295K mutation on the prechemistry barrier [1]. That is, the authors overlooked two key issues: the first is that if the barrier for the conformational change is actually rate limiting (as seems to be assumed by SABW but contradicted by their figure in the SI of Ref. [42]) then it presents no conflict with the concepts of Warshel and coworkers, which are very clearly related to the rate-limiting step, whereas the prechemistry and checkpoint proposals have very clearly invoked catalytic effects of non-rate-limiting barriers (see e.g., [4, 9, 43]). Miscommunicating this point can be problematic. Additionally, it is unclear how reliable is the calculated prechemistry conformational barrier, and why the effect of the mutation on the chemical barrier has not been elucidated. That is, any attempt to invoke prechemistry mutational effects *must* explore both the chemical and conformational barriers. Finally, there is no experimental evidence that the prechemistry barrier is rate determining.

The discussion of the mutation effects is shifted further from the relevant issue by the discussion of the Ref. [14]. That is, it is not entirely clear what the purpose of bringing up Johnson's work [14, 44] is, since it has no direct relevance to the prechemistry argument, but only to the case where the conformational barrier can be rate limiting. If the point is that "*In this situation, the flux through the catalytic cycle is significantly reduced due to the low concentration of ES. Since the magnitude of* k_{-2} *is not known in most instances, the observed reaction cannot be directly related to chemistry or conformational changes*", then we have a major problem. That is, any single element of the overall kinetics is uniquely and completely determined by knowing the activation barriers for each step (and thus all the elementary rate constants) and, of course, defining the relevant reactants concentrations. The difficulties in determining k_{-2} experimentally is common to the field and has little to do with the prechemistry argument. In fact, Johnson misunderstanding and complicated discussion of the back reaction reflect the confusion and difficulties of thinking on rate constants and kinetics instead of realizing that a known free energy landscape does completely define the kinetics.

2.8 On the need of calculations of many effects

SABW also bring up a presumed fundamental problems with correlating computationally determined barriers and observed kinetics stating that "*Computational methods are subject to the well-known approximations and imperfections of force fields and sampling*". This seems to reflects an unfamiliarity with the requirement form reliable calculations of activation free energy for the chemical steps, where in fact using proper reference solution reactions makes is possible to routinely obtain quantitative results once one is familiar with proper evaluation of electrostatic effects. In some cases, one can also successfully calculate various conformational barriers (e.g., [6]). Pointing out general problems rather than validation studies does not help at all. The fact that it is sometimes hard to experimentally determine k_{pol} is well known and should be overcome by different approaches that slow down the chemical step, and by using markers such as isotope effects (e.g., [45]). If this argument is meant to cast doubt on the validity of theoretical studies, then we have a major problem, since proper theoretical studies have been constantly validated on cases where the chemical barrier is

Table 1 The observed ($\Delta G^{TS}_{bind,obs}$) and calculated TS binding free energies ($\Delta G^{TS}_{bind,cal}$) for the incorporation of C and A opposite G

dCTP:G (correct pair)				dATP:G (wrong pair)		
Pol β variant	K_{pol}/K_d ($M^{-1}s^{-1}$)	$\Delta G^{TS}_{bind,obs}$ (kcal/mol)	$\Delta G^{TS}_{bind,cal}$ (kcal/mol)	K_{pol}/K_d ($M^{-1}s^{-1}$)	$\Delta G^{TS}_{bind,obs}$ (kcal/mol)	$\Delta G^{TS}_{bind,cal}$ (kcal/mol)
Wild type	1100000	−13.55	−14.49	77	−7.68	−10.72
R283A	4900	−10.24	−9.76	33	−7.16	−8.22

The observed TS binding free energies were determined from the respective catalytic efficiencies (K_{pol}/K_d) of pol β for non-gapped duplex DNA as substrate [67]

exactly known. We would also like to clarify that arguments about force fields or other computational problems are not useful. Theoretical methods in biophysics should be validated by careful comparison with experiment, by comparison with ab initio surfaces, and by convergence studies, and, as far as the EVB is concerned, it passed such tests long ago [24, 46–48], and its validity in determining activation free energies should not be challenged without pointing out real cases where it has not worked and where other approaches performed in a superior way.

In a complete contrast to the implications of SABW, the validity of computational analysis of the origins of catalysis *cannot* be experimentally explored. That is, calculations should reproduce all known relevant experiments about the rate acceleration, and, once this is done, calculations are the *only* way to dissect the different contributions to the observed barrier. All the assertions on the many computational approaches that are presumably needed for proper studies of enzymes overlook the fact that most of the approaches for studies of enzyme catalysis and function have been introduced by Warshel and coworkers, who also paved the way for exploring the validity of any possible catalytic effect and proposal. Thus, the misleading statement that "*A view that focuses on the chemical barrier overlooks important steps required to achieve a catalytically competent state and, accordingly, cannot reliably predict the behavior of altered enzymes*" is of particular concern. Calculations of the chemical barrier *must* focus on the chemical barrier and not on essoteric effects such as the speed of rotation of some side chain. Of course, one does not have to check the steps needed to reach a catalytic configuration to account for the (experimentally!) observed rate of the chemical step. And, again, it is mainly Warshel and coworkers who actually explored and established this fact (e.g., [13] and simelar conclusions have been reached by others (e.g. [49, 50]). So arguing that such approaches cannot predict the activity of altered enzymes is one of the most problematic parts of the SABW paper. The fact is that we have repeatedly predicted the activity of countless altered enzymes [24, 51–53], and we are not aware of any such prediction based on the dynamical concepts promoted by SABW and others. We would also like to clarify that it is easy to imply that all calculations are important in order to understand catalysis, and that this might be popular in some circles. However, studying biological function should focus on simulating the given function, and not on irrelevant observations (as we have demonstrated for a long time [54]. Thus, for example, approaches that reproduce the thermal expansion of the enzyme, or the normal modes of the enzyme or related factors are not going to aid our understanding of catalysis. Of course, exploring the incorrect proposal that pressure contributes to catalysis would require one to explore the pressure effect [55]. But, once it is demonstrated that pressure does not aid catalysis, we do not believe that approaches that simulate pressure effects will improve our understanding of catalysis and fidelity.

2.9 The relevance of conformational changes should never be confused with the relevance of conformational barriers

In an attempt to further support the prechemistry idea, we are again told that "*The observation that conformational changes are required for catalytic activation supports the concept of an induced fit mechanism proposed by Koshland and related mechanisms*". However, this statement has major logical difficulties. That is, conformational changes that lead to the ES do not and cannot account or tell us about the chemical catalysis. The only factor that counts is the free energy landscape of the final folded ES complex after the change occurs (and if we are interested in k_{cat}/K_M then the difference between the TS free energy and the energy of the unbound state). Of course, if the protein is unfolded, it would not have any catalytic ability, but this fact, as well as the fact that the presence of a substrate leads to a different structure than the one without the substrate, provides no information on the factors that control catalysis and cannot be used to understand or to logically explain fidelity. Furthermore, the argument that Post and Ray have shown [56] that "*the induced fit can alter enzyme specificity even when critical conformational changes are not rate limiting*" is problematic. That is, it is obvious that having different substrates may lead to changes in the ES structure, but this has nothing to do with SABW's arguments or with the rate-limiting issues. The barrier for the conformational change does not enter into any of the arguments of [56], rather, it is all about having different chemical barriers for different substrates (see Fig. 8 in [8]). We are also again told about the seemingly remarkable fact that there are substrate-induced conformational changes, as if we have any problem with this trivial fact.

Finally, we return to the argument that the entire profile and dynamics is needed in order to understand catalytic activity. We strongly disagree with this assertion, since the only factors needed to understand *true* catalytic activity are the factors that control the activation free energy of the rate-determining step. If the rate-limiting step is the chemical step, then we have to understand why the corresponding barrier is lower than the barrier for the reference reaction in aqueous solution. This is simply and absolutely the part where the largest catalytic effects have been optimized by evolution (see Fig. 2). Now if we have a rare case where the rate-limiting step is the conformational change, then not only is the difference between the conformational and chemical barriers small, but also, as far as

the catalysis is concerned, the difference between the rate-determining barrier and that of the uncatalyzed barrier (i.e., the barrier to the reference reaction) is similar to that between the chemical barrier and the uncatalyzed barrier (Fig. 2). Therefore, an understanding of catalysis can only be obtained by focusing on what the factors that reduce the chemical barrier are, and not why after nature has reduced the chemical barrier, the conformational barrier becomes slightly higher. Of course, it is challenging to use computational approaches to evaluate the conformational barrier, but this has little to do with understanding catalysis. In fact, we are not aware of a *single* case where the catalytic power of the enzyme was accounted for by evaluating the conformational barrier, nor do we expect to ever see such a case as long as the catalytic effect is large.

So as a summary to this section, we reemphasize that the real issue is what is the barrier height of the rate-determining barrier of the complete system (with the substrate) and not the trivial fact that the enzyme changes it structure from the unbound state to the ES. Of course the change of structure changes the chemical and other barriers. In fact, the barriers will have a limited meaning if they are not determined along a structural coordinate.

3 The meaning of Johnson's experiments and their relevance to the argument about the origin of fidelity

As stated above, SABW brought up Johnson's work [14, 44] in a way which is not directly related to the prechemistry argument. Furthermore, theses experimental studies were not obtained for pol β. Nevertheless, they are related to the general issue of the control of fidelity, in particular because of the implications that they present a new paradigm. Thus, it is useful to consider this issue in more details. However, since the main issues with Johnson's proposal were already discussed in Ref. [6], we will examine here a recent work [57] that performed elegant "Milestone" simulations of the conformational change in HIV reverse transcriptase and implied that DNA polymerases select the correct substrate by means of conformational dynamics. Now despite the technical accomplishments involved in the Milestone approach (noting, however, that our renormalization approach [58] has also provided a very good estimate of the conformational barrier [6]), we find the dynamical and conformational view to be problematic. That is, Johnson, Elber and their coworkers [57] examined a case that might involve a rate-determining conformational barrier for the formation of the correct base pair (Fig. 4, presents an identical case in T7 polymerase [44]). Now, the dynamical implications suffer from several problems. That is, first (as was clarified in [6]), there is nothing (apart from evolutionary constraints) forbidding a protein from having a rate-determining

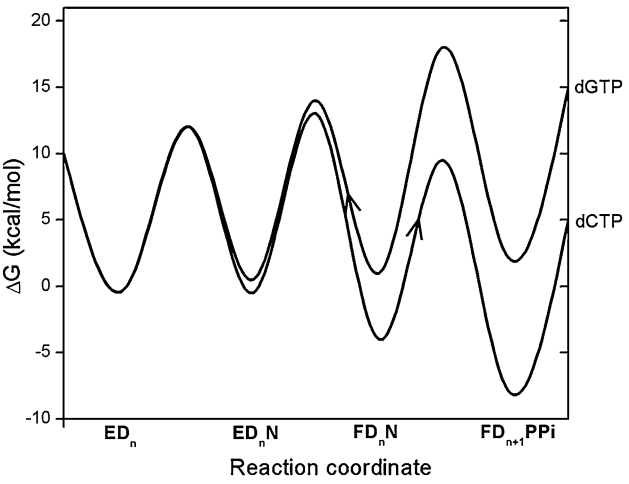

Fig. 4 A schematic free energy diagram for correct (green: dCTP) and incorrect (*red* dGTP) nucleotide incorporation reactions for the T7 DNA polymerase (adopted from Ref. [44]). Where EDn represents the enzyme–DNA complex with a primer strand n residues in length, N: the incoming dNTP, and FDnN: the closed state of the enzyme with bound DNA and nucleotide

conformational barrier. However, as we will explain below, this has very little to do with dynamics, and of course cannot be used as (even implicit) support of the checkpoint and prechemistry proposals, which have been clearly stated to be related to the specific case where the chemical barriers are rate limiting. Second, although the simulations reproduce the experimental conformational barriers, they have not considered the chemical barrier and thus have not established the origin of the fidelity. Furthermore, the calculated conformational barriers could not be correlated with the observed fidelity. We would also like to clarify (as was done in [6]) that even if the rate-determining barrier for the insertion of the correct dNTP substrate is the conformational step, this has nothing to do with the dynamical view that "*rapid motions in the specificity domain allow the enzyme to explore the substrate at the active site, probing for key interactions indicating a correctly bound substrate*", presented in [57]. Rather, all we have here is being entirely determined by the (conformation and chemical) activation barriers, and the depth of the binding free energy, which fully determines the kinetics of the system. The "speed" of the motion is neither remembered, nor used to climb the chemical barrier (that is, the system has enough time for fully stochastic motions at each of the minima, see also [13]). The statement that "*only the correct substrate induces a rapid and energetically favorable alignment of catalytic residues to bring reactants together at the active site*" [57] is as problematic as the proposals that the conformational motion is bringing the system to its preorganized structure [59] (see discussion in Fig. 7 of [7]). This fact has been established in works that *actually* explore the dynamical proposal (e.g., [13]) and has never been explored by other simulation studies. The correct

physical picture is, in fact, very simple. That is, for the incorrect dNTP substrate, the conformational barrier is lower than the chemical barrier, and therefore, the rate is fully determined by the height of the chemical TS (see Fig. 4), and the system will move over the chemical barrier according to the Boltzmann probability of this barrier. Furthermore, the system will also move *backward* to the ED_nN, over the conformational barrier, according to the Boltzmann probability of moving from ES to ED_nN. The chance of going backward across the barrier is a trivial kinetic effect of having a lower barrier than the chemical step and has nothing to do with dynamics. If it is true that the conformational barrier is rate limiting for the insertion of the correct dNTP substrate, the rate is determined by the Boltzmann probability of crossing the conformational barrier after the initial binding to the open state EDnN.

At this point, we note that the implication that the conformational barrier controls the fidelity is very problematic, since it is by far simpler to use the allosteric features depicted in Fig. 3 than to somehow have different conformational barriers for the correct (R) and incorrect (W) dNTP's. After all, evolution has had to work much harder to reduce the chemical barrier (from the very high barrier in the uncatalyzed reaction) than to change the almost trivial conformational barrier. In fact, the entire situation depicted in Fig. 4 reflects the physics clarified in Fig. 5: in the R case (Fig. 5a), the system tries to get the fastest reaction, by pushing the top of the chemical TS (relative to the unbound state) *down* to the level of the conformational barrier (note that the reduction of the TS barrier depicted in Fig. 4 is most likely an exaggeration). Now in the case of W, the fidelity is accomplished by pushing up the TS barrier, which can be done either by just destabilizing the TS, or by destabilizing both the TS and RS, as is the situation illustrated in the Fig. 5b. In fact, the conformational barriers are very similar for W and R, and the small increase in the conformational barrier is just a result of the destabilization of the ground state Michaelis complex, rather than the reason for any observed fidelity.

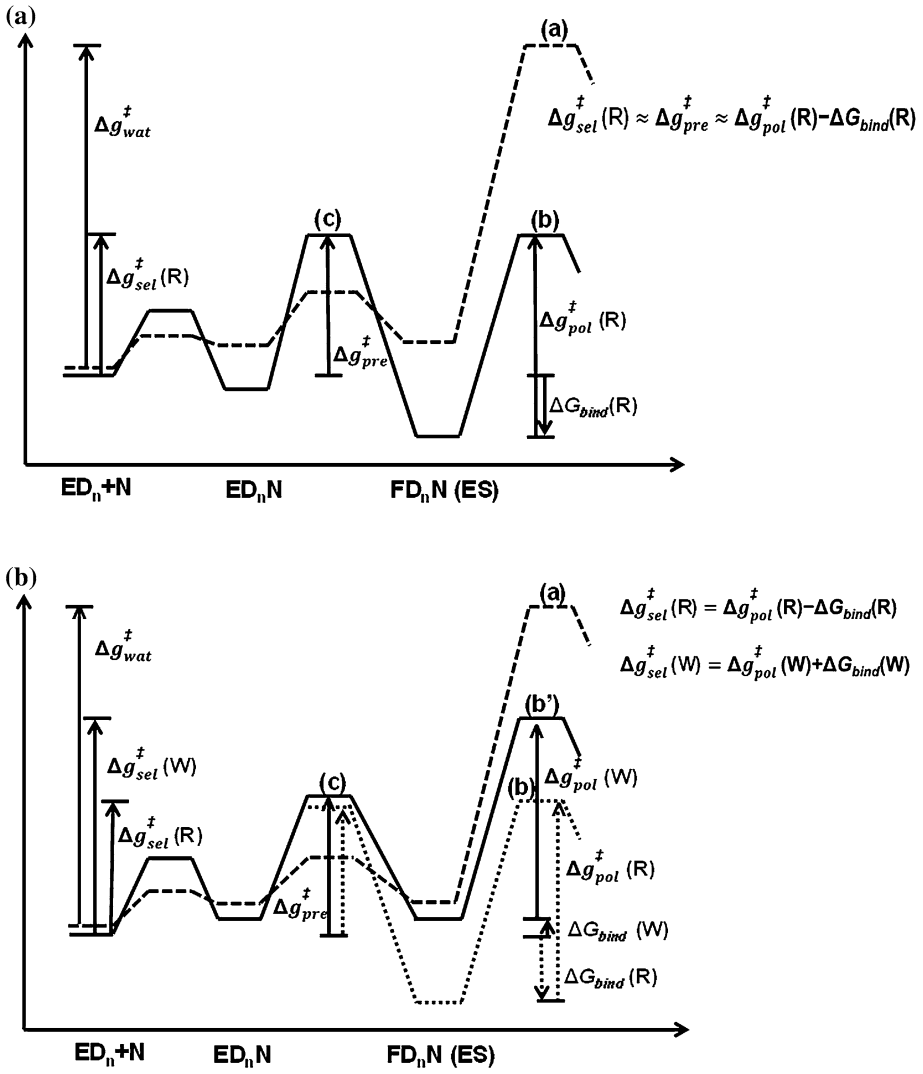

Fig. 5 Clarifying the evolutional control of replication fidelity. In the R case (**a**), the system tries to get the fastest reaction, by pushing the top of the chemical TS (relative to the unbound state) down to the level of the conformational barrier but there is no incentive to go below the diffusion limit. The solid and dashed lines represents respectively the free energy profiles of R in protein, and the corresponding solution reaction in water. Now in the W case of (**b**), the lower rate thank R (and thus the fidelity) is accomplished by pushing up the TS barrier, which can be done either by just destabilizing the TS, or by destabilizing both the TS and RS, as is the situation illustrated in the figure. The solid, and dashed lines represents respectively the free energy profiles of W in protein, and the corresponding solution reaction in water. The additional dotted line represents the free energy profile of R in protein. $\Delta g^{\ddagger}_{sel}$ designates the activation barrier for overall selectivity (see the definition in Fig. 5) that can, for example, be $\Delta g^{\ddagger}_{fidelity}$

4 Some crucial point that may have been overlooked in MRT meditating comments

A useful part of this exchange has been the mediation paper of Mulholland, Roitberg and Tunon (MRT) [60]. While we overall appreciate the commentary, and in particular the discussion of the dynamical issues, we have commented below on key critical issues about the meaning of the prechemistry proposal and related issues that we believe need clarifications.

In clarifying some misunderstandings, we are motivated by MRT suggestion that the readers should reach their own conclusions. In this respect, we note that one of the most important requirements when resolving scientific conflicts is a clear definition of the issues defining the different views. Avoiding doing so may be more politically correct, but it is also one of the main reasons for the long decay time of problematic scientific views such as the dynamical or low-barrier hydrogen bonding [61, 62] proposals. Thus, while we fully agree with [60] that the readers should make up their own mind about the prechemistry argument, we also believe that the typical busy reader, as well as readers that come from a more technical background (without the major experience necessary in order to follow arguments which are crucial for resolving biophysical issues) may not have the time necessary to do this. Therefore, it is essential to clarify (with proper quotations) what the actual controversies *are*. We will do so with regard to the main points.

As outlined in Fig. 2 of our manuscript, it is extremely important to distinguish between enzyme *catalysis* and enzyme *function*. That is, while enzyme function is the result of a complex multistep cycle, catalysis is, per definition, a rate acceleration, and it is what the enzyme has been evolutionarily optimized to facilitate. Unfortunately, "catalysis" is often used interchangeably particularly by biochemists to indicate both the actual rate acceleration, and also enzyme function. This distinction is clearly made in the beginning of MRT discussion, however, throughout the manuscript, in several places, there is semantic confusion where inadvertently these terms end up being interchanged which means that a non-specialist (or perhaps even non-chemist) would end up getting the wrong impression. This is particularly crucial on the first page, where the authors cite Warshel and coworkers as stating "strongly that dynamics makes no significant contribution at all [5, 6]". The authors do use "enzyme catalytic power" here, but it is extremely important to be careful in wording to not give the incorrect impression that Warshel believes that dynamics is also not *functionally* important (which is the impression one initially obtains from reading this). This is problematic also since Warshel have been the first to simulate functional dynamical effect in biology [63].

While we appreciate the conclusion that the free energy determines catalysis, we believe that the references given for this should include the works that actually clearly established this point (including Refs. [7, 13, 25, 26]. A similar problem arises at the point that cites the work of Ref. [64] for explaining the issues with simple transition state theory models for rationalizing the temperature dependence of kinetic isotope effects in some enzyme catalyzed reactions, while not mentioning the detailed study of Ref. [65], which clearly explored tunneling and dynamics in a number of systems (including lipoxegenase), and established the validity of TST. This issue is important since one of SABW main point is the presumption that we have not look at anything except the chemical barrier.

MRT seem to present a very serious misunderstanding, which may really confuse readers. That is, MRT present the entirely incorrect statement that "*the debate is based on the nature of the rate determining step*". This major misunderstanding seems to be based on the very long quotation from SABW [23] by MTR, where we have the quote that "*In the context of an induced fit model, the conformational change can be construed as a substrate selection gate. Initial "loose" binding of the correct substrate would trigger a conformational change leading to an active configuration of the enzyme where the key residues in the active site are brought into proper alignment, thereby providing transition state stabilization for catalysis…in contrast, binding of the incorrect substrates results in a bad fit*". As we have written many times, that *yes,* you do need correct folding, but it has nothing to do with SABW argument, and definitely nothing to do with the conclusion reached by MRT who assert that "*It is clear from this discussion that the potential importance of conformational changes depends on the relative rates of conformational and chemical changes*". This is problematic on two fronts. The first is that while it is not justifed to move from the discussion of the importance of correct folding for optimal transition state stabilization to arguing that then somehow the *rate* of the conformational change affects the rate of chemistry, but this is not even what SABW statement is saying. That is, of course if incorrect nucleotide insertion affects the conformational change in such a way that it results in a conformation with impaired TS stabilization (TSS), then this will detrimentally affect the rate of the chemical step, but this is simply due to lost active site preorganization and has *nothing* to do with the actual *rate* of the conformational change (as long as it is not rate determining). In addition to this, the subsequent passage completely overlooks the essence of all the controversy. In fact, Schlick and coworkers (as well as other workers (e.g., Ref. [9]) are crystal clear about their view that *non*-rate-determining conformational changes play a key role in determining the overall rate and fidelity. To prevent any further misunderstandings, we use as an example clear quotations the statements given in Sect. 1 rather the almost impossible interpretation of every quotation.

To be clear, there is no argument about the importance of *understanding* the conformational changes during the whole cycle of the enzymatic action. The central question is whether these conformational (or "pre-chemistry") events would contribute to *catalysis*, and then to the fidelity of DNA polymerases, if they are *not* rate limiting. As we stated (and was also pointed out by MRT, [60]), "As long as the free energy barriers associated with any of the pre-chemistry steps are not the rate limiting they could not contribute to the catalysis and then to the fidelity". Therefore, the dispute is *not* about the relevant rates of the conformational and chemical steps, but rather whether non-rate-limiting conformational ("prechemical") steps can actually influence the rate of the rate-limiting chemical step.

MRT discussion of Johnson's experiment [14, 44] is also problematic, although this is understandable in light of the complexity of the relevant issues, which are now discussed in Sect. 3. We are highlighting some of the main misunderstandings here. These start with the statement "*In the analysis presented by Warshel and coworkers the chemical step is assumed to be the rate-determining one*". It should be noted that it was Johnson who "assumed" that the chemical step is rate determining in his analysis [14]. We demonstrated that it is, through careful calculations, and therefore "assume" here is somewhat misleading. The more problematic issue is, however, the fact that it is implied that there is no contradiction between the two points of view (which at present reads initially as if the authors are talking about the proposals of Warshel and Schlick and coworkers, which are fundamentally orthogonal, rather than the intended discussion of differences between the proposals of Warshel and Johnson). But even the idea that there are similarities between Warshel and Johnson's proposals reflects major misunderstandings, as outlined in Sect. 3. In fact, the different in point of view is fully reflected by the title of the new work of Johnson [57] where it is suggested (or implies) that in case where the conformational barrier is higher that the chemical barrier the control of fidelity is dynamical effect, while we maintain that even if the conformational barrier is rate limiting (only for R) this simply reflects a higher free energy barrier.

Finally, we must clarify the misunderstanding in MRT statement that "*the pre-chemistry steps are required for the chemical step and the function of the enzyme can only be explained according to the structures found through the whole catalysis cycle*". Unfortunately, as we have stated repeatedly, if the prechemistry step has a lower barrier than the chemical step, then understanding it's nature is not needed for understanding the function of the enzyme and, in particular, not for understanding catalysis. Now clearly in a situation in which the conformational change is required to bring the enzyme to the correct preorganized state, then blocking this would impair function, which would in turn impair catalysis. However, subtle pre-chemical conformational changes *do not* prevent arriving to the preorganized state unless they also change the ES structure, which was never an option in the discussion here. This would basically be a throwback to dynamics people who always interpret distanced mutations as changing dynamics, whereas they merely change the active site preorganization by equilibrium property (see our clear demonstration of this Ref. [26] when addressing the incorrect argument that there are "dynamical knockout mutations" of DHFR that restrict the flexibility of the active site residues and mildly impair catalysis, thus "proving" the role of dynamics in chemical catalysis [19]). It should be very clear that we (and also Schlick and coworkers) are talking about changes of the *path* to the ES and never about changes to the ES *structure*. Here, all the controversy can be avoided simply by stating that all steps must in principle be understood and that Warshel and coworkers clearly state that while the prechemistry step is interesting, there is no *need* to understand it in order to understand catalysis, *if* this step is not rate-limiting.

5 Concluding remarks

At this point, we feel that it might be useful to re-emphasize several key points:

1. The entire argument should be restricted to prechemistry and checkpoint barriers that are not the rate-limiting step. Any case where the prechemistry barriers are higher than the chemical barrier may be interesting, but they are irrelevant to the current discussion.
2. We do not believe that any information about the chemical barrier (and the rate of the chemical step) can be obtained by examining the prechemistry barrier, nor we are aware of such an analysis.
3. Bringing up cases where the prechemistry is rate limiting is completely misleading as far as the current debate is concerned.
4. Bringing up the fact that large structural changes are involved is irrelevant, as we all agree on this fact. The disagreement is rather about whether the chemical step carries a memory of the conformational change.
5. Invoking mutational effects as a proof for prechemistry effects without exploring the obvious effect of the mutations on the chemical step is not useful.
6. The idea that all the properties of the protein must be studied in order to understand catalysis is counterproductive, when it is related to properties that have no

effect on the chemical barrier. In fact, it is simply not so useful to explore all irrelevant properties while not studying the most relevant issue namely the chemical barrier when studying enzyme catalysis.

At this point, we feel it is crucial to take exception to the concluding assertion of SBAW that "*a view that focuses on the chemical barrier* [14] *overlooks important steps required to achieve a catalytically competent state and, accordingly, cannot reliably predict the behavior of altered enzymes*". Not only that focusing on the chemical barrier in studying chemical rates is much more useful than looking at all factors except the chemical barriers, but presuming that we have not studied or cannot explain altered enzymes is at best extremely misleading. Not only that our studies of mutational effects in polymerase have been far more reliable than that reported by others [24] but one of us have introduced calculations of mutational effects [51] and have reported countless quantitative studies of mutational effects (e.g., Ref. [52]).

Finally, we would like to point out that if prechemistry steps are rate limiting, we have to be aware of this fact (although it is not related to the prechemistry idea). However, this situation does not provide a useful way of controlling fidelity (e.g., see Ref. [66]).

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