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New Concepts

A Reexamination of the Nucleotide Incorporation Fidelity of DNA Polymerases†

Alexander K. Showalter‡ and Ming-Daw Tsai*,‡,§

Departments of Chemistry and Biochemistry, The Biophysics Program, and The Ohio State Biochemistry Program, The Ohio State University, Columbus, Ohio 43210

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ABSTRACT: Intensive study has been devoted to understanding the kinetic and structural bases underlying the exceptionally high fidelity (low error frequencies) of the typical DNA polymerase. Commonly proposed explanations have included (i) the concept of fidelity check points, in which the correctness of a nascent base pair match is tested at multiple points along the reaction pathway, and (ii) an induced-fit fidelity enhancement mechanism based on a rate-limiting, substrate-induced conformational change. In this article, we consider the evidence and theoretical framework for the involvement of such mechanisms in fidelity enhancement. We suggest that a "simplified" model, in which fidelity is derived fundamentally from differential substrate binding at the transition state of a rate-limiting chemical step, is consistent with known data and sufficient to explain the substrate selectivity of these enzymes.

The nature of DNA replication fidelity is of immense biological importance due to the fundamental requirement for accurate DNA reproduction, in both replicative and repair processes, to the propagation of life. It is also of great interest to the mechanistic enzymologist because of the seemingly difficult challenge with which a DNA polymerase is faced in fulfilling this requirement. A polymerase is presented with a pool of four structurally similar deoxynucleotide triphosphates (dNTPs) from which it must select the sole correct (i.e., Watson−Crick base paired) substrate for incorporation into the growing DNA strand. Moreover, the identity of the correct dNTP changes with each cycle of nucleotide incorporation, as translocation leads to the presence of a new "templating" base. Despite this challenge, most DNA polymerases are extremely accurate, with error frequencies for nucleotide incorporation ranging from 10^{-3} to 10^{-6} (*1*, *2*). In contrast to this superb enzymatic selectivity, it has been shown that a Watson−Crick base pair is only ∼0.2−4 kcal/mol more stable than a mismatched base pair in free solution (*3*). A free energy difference of this magnitude, if expressed at the chemical transition state of a nucleotide incorporation reaction, would give rise to differences in catalytic efficiencies between correct and incorrect base pair incorporations of ∼2-fold to a few hundred-fold.

Current Model for DNA Polymerase Fidelity

The question of DNA polymerase fidelity then could be stated as follows: What mechanistic and structural properties could enable a polymerase to catalyze nucleotide incorporation with a selectivity far greater than that which is dictated by the thermodynamic differences between base pairs in free solution? Because of the importance of this question, a great deal of study has been dedicated to providing an answer, and a discrete number of widely cited concepts have emerged. Of necessity, such concepts have evolved along

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* To whom correspondence should be addressed at the Department of Chemistry. Telephone: (614) 292-3080. Fax: (614) 292-1532. E-mail: tsai.7@osu.edu.

‡ Department of Chemistry.

§ Department of Biochemistry, The Biophysics Program, and The Ohio State Biochemistry Program.

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with an elucidation of the elementary steps of the reaction, which include, through the chemical step, (i) initial binding of Mg·dNTP into an "open" complex, (ii) a global conformational change, induced by the initial binding event, to a "closed" complex, (iii) binding of a free, "catalytic" Mg^{2+} ion, and (iv) nucleotidyl transfer.

A commonly cited model is embodied in the statement that, "There are at least three possible check points for proper geometric alignment during base insertion by polymerases: initial dNTP binding, postbinding selection for the correct geometry by an induced-fit mechanism, and the chemical step of phosphodiester formation. Previous data suggest that there are significant differences in the extent to which different polymerases use each of the check points" (*4*). The broad acceptance of this model has been reiterated and reinforced in a number of recent reviews (*2*, *5*).

Problems with the Current Model

The model described above consists of two important concepts, those of the induced-fit (Koshland's hypothesis) and fidelity check points, which in our view do not accurately explain the fidelity of DNA polymerases. In the following discussion, we will consider these two concepts in light of what is known about the elementary steps (principally steps i, ii, and iv, since step iii has largely been ignored in the description of these concepts), and in so doing arrive at an alternative description of the root cause of polymerase fidelity. It is important to point out that there is no argument with the suggestion that DNA polymerases can distinguish between correct and incorrect dNTP at multiple elementary steps, and that the degree of discrimination at various steps can (and does) vary among different enzymes. Such stepwise discrimination is supported, for example, by basic kinetic data from many labs, including our own. The key issues being addressed in this paper are which step is rate-limiting and how (or whether) discrimination at each specific step contributes to the overall fidelity.

Induced-Fit Mechanism for DNA Polymerases

An important feature in the current model is the nucleotide-induced conformational change (*6*), or induced-fit (the second elementary step in the process). The theory suggests that a substrate-induced conformational change, which is induced preferentially by the favored substrate and activates the enzyme for catalysis, can increase the enzyme's selectivity. Fersht has argued (*7*) that such a mechanism cannot enhance an enzyme's substrate selectivity, while others have argued (*8*, *9*) that, under certain circumstances, it may.

Comparison of the crystal structures (*10*−*12*) of the binary polymerase·DNA complex with the corresponding ternary polymerase·DNA·dNTP complex (with the correct incoming dNTP and two metal ions) demonstrates that correct nucleotide binding induces a conformational transition from a relatively open form to a more closed form, producing a thermodynamically stable complex and aligning active site residues for chemistry. This conformational change, presumably (*13*) by being induced preferentially by binding of correct dNTP as opposed to incorrect dNTP, is commonly thought to constitute an induced-fit fidelity enhancement mechanism of the variety envisioned by Koshland (*14*). An important issue related to this concept is whether the conformational change is rate-limiting, which is discussed below.

What Is the Rate-Limiting Transition State?

According to the transition state theory of chemical reaction rates, as applied to enzyme-catalyzed reactions, the pseudo-second-order rate constant, k_{cat}/K_m (or in the pre-steady state, $k_{pol}/K_{d,app}$), by which substrate specificity is defined, is a direct function of the free energy difference between the initial state and the most unstable transition state. This implies that it is the highest-energy transition state in the reaction pathway (the transition state for the rate-limiting step) which directly dictates catalytic efficiency as well as fidelity. The latter results from the free energy difference between the highest-energy transition states of the two competing reactions. Thus, the substrate-induced conformational change (or any other step) is not directly responsible for the enzyme's fidelity if it is not rate-limiting.

Significantly, it has been proposed that the substrate-induced conformational change is the slowest step, and therefore predominantly rate-limiting, in the nucleotide incorporation reaction (*15*, *16*). When the reactions for incorporation of correct and incorrect nucleotide are both considered, which they must be for fidelity to be evaluated, it is commonly suggested that the substrate-induced conformational change is completely rate-limiting for correct nucleotide incorporation but only partially rate-limiting (*6*), or not rate-limiting (*17*), for incorrect nucleotide incorporation. In other words, the commonly stated induced-fit proposal for a DNA polymerase focuses on a nucleotide-induced conformational change which limits the rate of correct nucleotide incorporation to a greater extent than it limits the rate of incorrect nucleotide incorporation.

This contention has been based principally on kinetic studies in which a nonbridging oxygen on the α-phosphate of the incoming dNTP (usually the *pro*-S_p oxygen, but sometimes a diastereomeric mixture is used) is replaced with a sulfur atom. It is generally thought that a nonbridging sulfur atom, due to a decreased electronegativity, is less effective than oxygen at inductively stabilizing the accumulation of electron density at an associative transition state for phosphoryl transfer. The magnitude of this "thio effect" (the reaction rate with the natural substrate divided by the reaction rate with the thio-substituted substrate) is then taken as being diagnostic of whether chemistry is rate-limiting. If chemistry is rate-limiting, then the full thio effect will be observed, whereas if another step, insensitive to thio substitution, is rate-limiting, then the thio effect will be diminished. The thio effect for DNA polymerases is usually small for correct nucleotide incorporation but significantly greater for incorrect nucleotide incorporation.

The reliability of the thio effect for determining whether chemistry is rate-limiting however is debatable, as has been previously noted (*18*, *19*). It is based on the assumption that the thio effect of an enzyme-catalyzed reaction can be directly compared to that of a free solution reaction thought to have a comparable transition state. This requires that the nature (i.e., associative, dissociative, or intermediate) of the chemical transition state in the enzyme active site be known, which it often is not. If one assumes that the transition state of phosphoryl transfer in the polymerase active site is purely

associative (for both correct and incorrect nucleotide incorporations), can it be further assumed that the "inherent" thio effect for this step will be in the range of 30−100 as has been observed for associative phosphoryl transfer reactions occurring in free solution (*20*)? We believe that it cannot.

The free solution reaction occurs in an achiral environment, and thus, all nonbridging oxygens on the central phosphate make an equal inductive contribution to stabilization of the associative transition state. In the polymerase active site, however, metal ion coordination occurs exclusively with the *pro*-R_p nonbridging oxygen (*10*). This would likely have the effect of causing transition state stabilizing inductive electron withdrawal to flow predominantly toward the *pro*-R_p oxygen, to the exclusion of the *pro*-S_p oxygen, thereby making the latter a less significant factor in transition state stabilization relative to its role in the achiral free solution reaction. Since the polymerase almost exclusively incorporates the (S_p)-thionucleotide (*21*, *22*), one should expect a decreased thio effect for the enzyme-catalyzed reaction in proportion to the degree that inductive electron flow is biased by the coordinated metal ions. Such a model suggests that a misleadingly small thio effect can be observed, due to the stereochemically absolute positioning of the catalytic metal ions, even if chemistry is fully rate-limiting. In brief, we would suggest that the thio effect should not be used as a determinant of which step is rate-limiting, but that more direct means should be used instead.

As an example of this, single-turnover stopped-flow fluorescence studies (*23*) of correct nucleotide incorporation by mammalian DNA polymerase β (Pol β) show two detectable fluorescence transitions, one preceding and one following the chemical step [with intervening metal binding/release steps which are so rapid that they are kinetically unobservable (*23*, *24*)]. Crystal structures of intermediates in the polymerization reaction pathway have been obtained by using an exchange-inert Cr·dNTP complex [exchange-inert in this context means that the metal·nucleotide complex is kinetically stable (*25*), and thus can be added to the enzyme without the presence of free metal ion which is required for the chemical step]. Such studies, along with additional stopped-flow studies using a dideoxy-terminated primer (*23*) or Cr·dNTP (*24*), clearly identify the first of these two fluorescence transitions as corresponding to the nucleotide-induced subdomain closure described above (and the second as corresponding to a later subdomain reopening step), and argue strongly against the existence of any kinetically or thermodynamically significant "micro" conformational change steps, as has been previously suggested (*26*, *27*). Most importantly, the rate of the prechemistry fluorescence transition, a direct observation of the rate of the substrate-induced conformational change, is ~5−10 times greater than the rate of nucleotide incorporation, which is therefore almost certainly limited in rate by the chemical step (*23*). Further studies of correct nucleotide incorporation using the exchange-inert Rh·dNTP complex (unpublished results) corroborate these findings, showing that the reaction when initiated from the starting point of a conformationally closed ternary complex occurs at the same rate as the reaction when initiated from the starting point of a conformationally open binary complex, indicating that subdomain closure is not rate-limiting.

While Pol β is a repair polymerase and has been described by some as a low-fidelity enzyme, its basic kinetic properties [including, in particular, thio effects for correct and incorrect nucleotide incorporation (*28*)] are very similar to those of other polymerases. In addition, in contrast to truly low-fidelity enzymes discovered recently (*29*−*33*), it is clear that Pol β, with error frequencies ranging from $<10^{-3}$ to 10^{-5} (*28*, *34*), possesses a kinetic mechanism which strongly selects for correct nucleotide incorporation. Nonetheless, studies of the type mentioned above should be repeated with other polymerases to determine whether the dNTP-induced conformational change or the chemical step is rate-limiting in other systems.

Effect of a Rate-Limiting Conformational Change: Free Energy Diagrams

In the previous section, we argued that the fundamental evidence for a rate-limiting conformational change in the DNA polymerase reaction pathway is fairly weak, and presented more direct evidence for one polymerase (Pol β) that there is no rate-limiting conformational change. Independent of such experimental evidence, we now consider the effect of a rate-limiting conformational change, in multiple formulations, for the fidelity of any polymerase. Figure 1 shows four possible situations, where the rate-limiting steps for correct and incorrect dNTP are conformational change and chemistry, respectively (A), chemistry and conformational change, respectively (B), conformational change for both (C), and chemistry for both (D).

Figure 1A represents the commonly accepted model mentioned above. In this model, rather than retaining the catalytic efficiencies and the fidelity inherent in the competing chemical transition states, the enzyme selectively reduces the efficiency of correct nucleotide incorporation and thereby decreases its own fidelity. Any extent to which the conformational change is more rate-limiting for correct nucleotide incorporation than for incorrect nucleotide incorporation directly causes a fidelity decrease. Thus, this commonly proposed model for polymerase fidelity cannot be viewed as an effective fidelity enhancer, because it would in fact have the opposite effect.

Alternatively, the polymerase could have a conformational change which is rate-limiting for incorrect but not for correct nucleotide incorporation, as illustrated in Figure 1B. This situation has not to our knowledge been proposed, but there is no direct evidence to support or disfavor this mechanism. As examination of Figure 1B reveals, the conformational change in such a mechanism would increase the overall fidelity relative to that inherent in the competing chemical transition states, by selectively reducing the rate of incorrect nucleotide incorporation. While the conformational change in such a mechanism has the ability to augment polymerase fidelity, we believe that this situation is unlikely to occur. Since the chemical transition state is the one in which the reactants themselves are likely to be the least stable (due to extreme distortion of covalent bonds), to stabilize this state the enzyme must bind to it with maximum affinity. This would seem to imply that the enzyme has the maximum number of specific interactions with the substrate at the chemical transition state. This is particularly likely in the case of a DNA polymerase, where binding of the catalytic

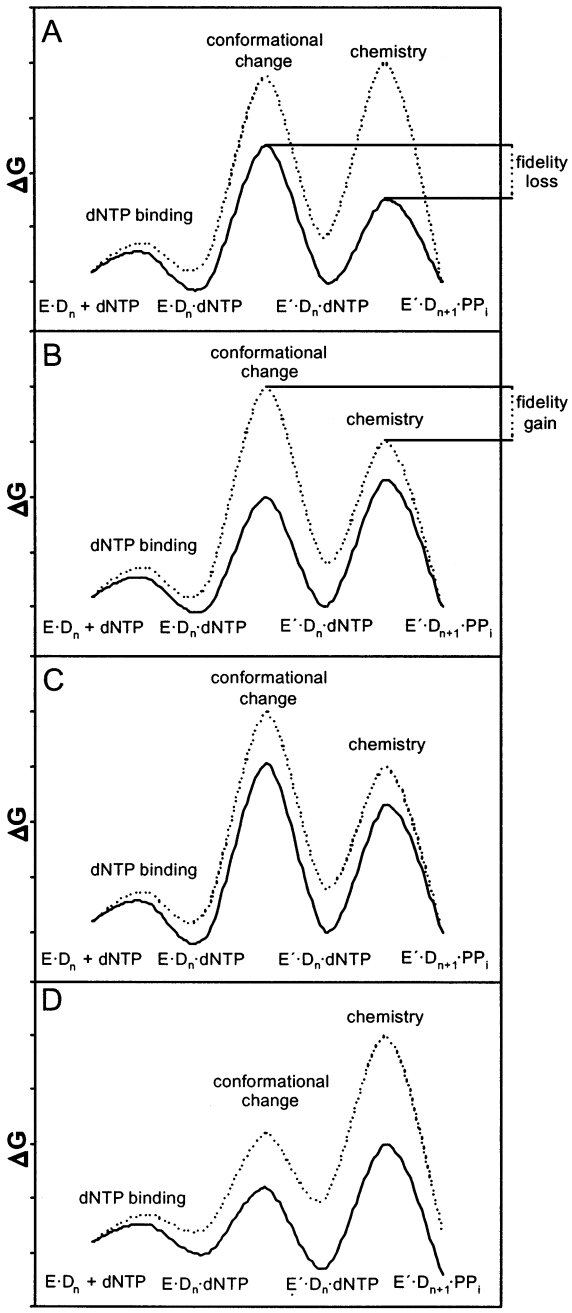

FIGURE 1: Free energy profiles of contrasting models for the polymerase conformational change. In all panels, the solid line represents correct nucleotide incorporation and the dotted line represents incorrect nucleotide incorporation. In panel A, nucleotide binding and chemistry are separated by a conformational change which is rate-limiting for correct nucleotide incorporation but not for incorrect nucleotide incorporation. The line labeled "fidelity loss" represents the amount of free energy by which the catalytic efficiency for correct nucleotide incorporation is selectively decreased. In panel B, the conformational change is rate-limiting only for incorrect nucleotide incorporation. The line labeled "fidelity gain" represents the amount of free energy by which the catalytic efficiency for incorrect nucleotide incorporation is selectively decreased. In panel C, the conformational change is rate-limiting for both competing processes, and in panel D, the chemical step is rate-limiting for both processes.

metal ion to form the complete Michaelis complex occurs immediately before the chemical step and after the nucleotide-induced conformational change (*23*, *24*). Thus, we would suggest that it is unlikely that the enzyme makes a greater distinction between correct and incorrect substrate in a step which has fewer specific enzyme−substrate interactions (the conformational change transition state) than in a step which has the maximum number of enzyme−substrate interactions (the chemical transition state), although this possibility cannot be formally excluded.

In Figure 1C, a mechanism in which the conformational change is rate-limiting for both correct and incorrect nucleotide incorporation is shown. This mechanism is subject to the same arguments applied to panels A and B, depending on whether the conformational change is more rate-limiting for correct or incorrect nucleotide incorporation. If the conformational change decreases the rate of correct nucleotide incorporation more than it decreases the rate of incorrect nucleotide incorporation (relative to the respective chemical steps), then it results in a fidelity decrease as in Figure 1A. If the conformational change decreases the rate of incorrect nucleotide incorporation more than it decreases the rate of correct nucleotide incorporation, it will increase the fidelity, but this also implies that a step at which the maximum number of enzyme−substrate interactions have not yet been realized has the greatest capacity to discriminate between competing substrates. As discussed in relation to Figure 1B, we believe that this is unlikely.

Figure 1D illustrates the final possible situation, in which there is no rate-limiting conformational change and thus no direct induced-fit fidelity enhancement. In this mechanism, the fidelity results directly from the difference in free energy between the competing chemical transition states. For the reasons described above, we feel that this most effectively accounts for high-fidelity properties of DNA polymerases. In the remainder of this discussion, we further consider the role that non-rate-limiting steps may play in contributing to the overall fidelity of the polymerization reaction.

Is Fidelity Controlled by Multiple Check Points?

As described above, the concept of fidelity check points suggests that the correctness of the nascent base pair is "tested" at different points along the reaction pathway, presumably implying that each check point contributes to the overall fidelity of the reaction. Thus by such a model, the "partial fidelities" apparent in initial nucleotide binding, the conformational change, and the chemical step in Figure 1D all make a contribution (either additively or multiplicatively) to the observed polymerase fidelity. The sequential application of check points has been suggested to be the source of the enzyme's exquisite sensitivity to any incorrectness in the nascent base pair.

The transition state binding theory of enzyme catalysis (*35*), in its barest essentials, includes the following concepts. (i) An enzyme evolves to bind specifically to the chemical transition state of the reacting species to be catalyzed. (ii) Catalysis results fundamentally from a tight binding interaction between the enzyme and reactant(s) at the chemical transition state. (iii) The degree of selectivity between competing substrates is a result of the stability of the reactants themselves at their respective chemical transition states, coupled with the binding affinity which the enzyme possesses toward those competing transition states. On the basis of this simple, yet in our view largely accurate, description of enzyme catalysis, we suggest an alternative view of the relationship between partial discrimination at individual steps

and the overall reaction fidelity. When Figure 1D is being considered, the discrimination between substrates which occurs at the initial binding step, for example, does not indicate that dNTP binding is a check point which augments the overall reaction fidelity. This difference is a consequence of the fact that the correct base pair, even in an early ground state, resembles the enzyme's preferred binding partner (the chemical transition state of a Watson−Crick base pair) to a greater extent than does the incorrect base pair. Similarly, as the complex approaches the chemical transition state in a stepwise manner (for example, by undergoing subdomain closure), the free energy difference between a correct and an incorrect base pair increases, due to the latter's relative inability to complement the structure of the enzyme active site. By this view, the fact that the structure of the enzyme's active site, particularly in its closed conformation, is intrinsically complementary to the chemical transition state of a Watson−Crick base pair is the source of all other properties, including the appearance of partial fidelities at individual steps. While this fundamentally represents an alternative manner of describing the same phenomenon (partial fidelities within individual elementary steps), we believe that it more accurately emphasizes the true source of polymerase fidelity.

Is Transition State Binding Sufficient To Explain the Fidelity of DNA Polymerases?

As outlined above, the "problem" of DNA polymerase fidelity lies in the apparent difficulty of selecting the one correct deoxynucleoside triphosphate, which changes with each cycle of nucleotide incorporation, from a pool of four structurally and chemically similar potential substrates. The problem is experimentally illustrated by the thermodynamic studies of Watson−Crick and mismatched base pairs mentioned above, which showed that the free energy differences between various base pairs within DNA in free solution could not account for polymerase fidelity.

Such a thermodynamic analysis poses a problem, however, only if one assumes that the free energy differences between Watson−Crick and mismatched base pairs in free solution are similar to the free energy differences between the same base pairs when bound at the enzyme active site and at the transition state for nucleotidyl transfer. This is almost certainly not the case. For example, it has been suggested that exclusion of water from about a base pair can amplify the free energy differences between Watson−Crick and mismatched base pairs to a level nearly compatible with polymerase fidelity (*36*). This suggests that binding to the enzyme active site greatly increases the free energy differences between correct and incorrect base pairs. Further, the intrinsic free energy differences between Watson−Crick and mismatched base pairs are probably substantially higher at the transition state for nucleotidyl transfer, where the alignment of reacting groups is mandatory, than in the ground state where no such requirements exist. For these reasons, free energy differences between ground state base pairs located in free DNA molecules are not a good comparative model for evaluating polymerase fidelity. Thus, it appears to be feasible that the strong preference for incorporation of a Watson−Crick base paired dNTP results from the fact that it is "simply the one for which the enzyme possesses stronger forces of attraction in the [chemical] transition state" (*37*).

Proposed Model

Earlier we concluded that Figure 1D is the most probable thermodynamic model for polymerase catalysis of competing base pair incorporations. Here we propose a full interpretation of the catalytic events, based on a rate-limiting chemical step and the absence of fidelity check points. First, initial nucleotide binding to the open ternary complex occurs rapidly, and is likely to be relatively nonspecific to correct or incorrect dNTP. Next, a non-rate-limiting, nucleotide-induced conformational change toggles the enzyme between the nonspecific open form, which is capable of substrate binding/release, and the closed form, which is capable of catalyzing the chemical step. The conformational change step is required because the open form of the enzyme is incapable of catalysis while the closed form is inaccessible to substrate in free solution (*38*). The significant energetic difference between Watson−Crick- and mismatch-containing complexes during the conformational change does not create reaction fidelity; it is a consequence of the approach to the chemical transition state, at which the maximum energetic difference exists. Finally, the rate-limiting chemical step occurs. The free energy difference between the competing, rate-limiting chemical transition states dictates fidelity.

As with any model, a critical determinant in evaluating this one will be consistency between prediction and future results. In interpreting such results, we suggest the following considerations. (i) From a structural standpoint, it is important to consider not only the properties of the Watson−Crick base pair which allow it to adopt the enzyme's "ideal" transition state but also the properties of the mismatched base pair (possibly including base pair hydrogen bonding) which prevent it from doing so. (ii) Since the free energy difference between correct and incorrect base pair reaction trajectories increases as the complex approaches the chemical transition state, intermediate species for the mismatch reaction are likely to be relatively unstable. For example, the qualitative free energy profiles in Figure 1D suggest that, for incorrect dNTP incorporation, the closed form of the ternary complex ($E'\cdot D_n\cdot$dNTP) is thermodynamically unstable relative to the open form ($E\cdot D_n\cdot$dNTP). This predicts the unlikelihood of crystallizing a mismatch-containing polymerase in the closed complex, as well as potential difficulty in spectroscopic detection of the closed complex during a mismatch incorporation reaction. (iii) Variation is to be expected among the mismatches. Thus, while the closed complex may be very difficult to detect for one mismatch incorporation, it may prove to be more feasible for another.

Recapitulation

We have argued three central points. (i) There is stronger evidence for a non-rate-limiting conformational change than for a rate-limiting conformational change in the kinetic pathway of DNA polymerases. In addition, the polymerase induced-fit fidelity enhancement mechanism, as it is commonly described in the literature, would actually decrease fidelity. (ii) The concept of fidelity check points, while being one way to describe the phenomenon of early-step, partial discrimination, masks the root cause of polymerase fidelity. (iii) This root cause is the specific binding affinity which the polymerase has for a Watson−Crick base pair at the chemical transition state.

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