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DNA Polymerase Fidelity: Kinetics, Structure, and Checkpoints†

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ABSTRACT: On careful examination of existing kinetic data for correct and incorrect dNTP incorporations by a variety of DNA polymerases, it is apparent that these enzymes resist a unified description. Instead, the picture that emerges is a rather complex one: for most polymerases, there is evidence for a noncovalent step preceding phosphoryl transfer, but there are less reliable data for determining whether the noncovalent step or phosphoryl transfer is rate-limiting during misincorporation. Although the structural conservation in the polymerase superfamily is probably reflected in a common set of intermediates along the reaction pathway, the energetics of these species vary even when closely related polymerases are compared. Consequently, some polymerases apparently show more discrimination between correctly paired and mispaired dNTPs in the binding step, and polymerases may differ in terms of which step of the reaction is rate-limiting in correct and incorrect insertion reactions. Because of the higher energy barrier in the misincorporation reaction, at least some of the intermediates both before and after the rate-limiting step in the misincorporation pathway will have higher energies than the corresponding intermediates in correct incorporation; consequently, these steps can serve as kinetic checkpoints.

Ever since it became possible to measure the fidelity of DNA polymerases, biochemists have sought to understand the source of the amazing specificity of these enzymes, which insert a nucleotide complementary to the templating base with an accuracy far surpassing what would be expected on the basis of the energetics of base pairing (*1*). Structural studies of DNA polymerases in ternary complexes with a DNA primer−template and the next correct dNTP illustrate the close steric complementarity between the enzyme active site and a Watson−Crick base pair, as well as the presence of hydrogen bonds between the protein and the minor groove side of the nascent base pair (*2*−*6*). Both these features could promote fidelity by excluding incorrectly paired dNTPs from the active site. Moreover, the snugly fitting active site could serve to exclude water from the vicinity of the nascent base pair, thus amplifying energetic differences between correct and mispaired nascent base pairs (*7*). A variety of steps along the reaction pathway could be envisaged as acting as "kinetic checkpoints", serving to test the incoming dNTP for complementarity and facilitating rejection of incorrectly paired dNTPs. One obvious candidate for such a checkpoint is the subdomain movement inferred by comparison of binary (Pol−DNA) and ternary (Pol−DNA−dNTP) complex crystal structures for several polymerases (*4*, *6*, *8*−*11*). This movement of the fingers (dNTP binding) subdomain interconverts the open and closed conformations of the poly-

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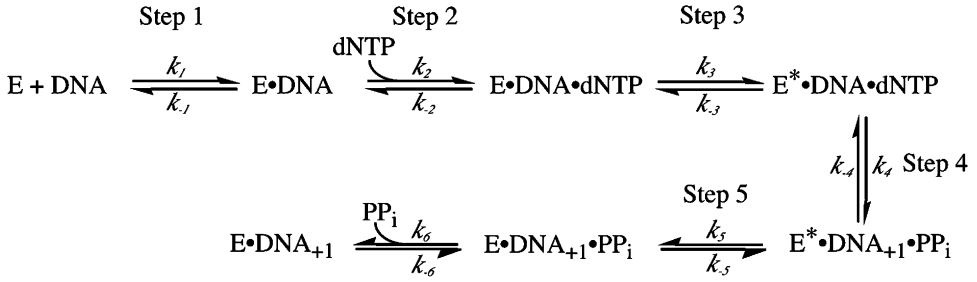
Table 1: Kinetic Data Used To Assign the Rate-Limiting Step in Correct and Incorrect dNTP Incorporations by DNA Polymerases

	correct dNTP			incorrect dNTP		
polymerase	pulse−chase/pulse−quench[a]	inference	ref	dNTPαS elemental effect[b]	inference	ref
Klenow fragment	pulse−chase > pulse−quench	$k_3 < k_4$	*17*	13	$k_3 > k_4$	*18*
T7 DNA pol	pulse−chase > pulse−quench	$k_3 < k_4$	*19*	20−40	$k_3 > k_4$	*20*
T4 DNA pol	EDTA quench = HCl quench	$k_3 > k_4$[c]	*29*	2	$k_3 < k_4$	*30*
HIV-RT	pulse−chase > pulse−quench	$k_3 < k_4$	*26*	1−3	$k_3 < k_4$	*31*
Pol β	no data			9	$k_3 > k_4$	*24*
Pol η	pulse−chase > pulse−quench	$k_3 < k_4$	*27*	2	$k_3 < k_4$	*27*
Dpo4	pulse−chase > pulse−quench	$k_3 < k_4$	*28*	6	$k_3 > k_4$	*28*

[a] Comparison of product yields obtained using pulse−chase or pulse−quench conditions. [b] Reaction rate observed with the natural (all-oxygen) substrate divided by that observed with an α-thio-dNTP. [c] See further discussion in the text.

Scheme 1



merase domain and forms the binding site for the nascent base pair. Structural studies suggest that the Y-family (lesion-bypass) DNA polymerases may be an exception. Comparison of unliganded and ternary complex crystal structures indicates little or no relative movement of subdomains, and invites speculation that this may be related to the low fidelity of these enzymes (*12*−*14*).

On the basis of their studies of DNA polymerase β (Pol β),[1] Showalter and Tsai (*15*) have raised another question: have kineticists made the DNA polymerase reaction mechanism more complex than is necessary to account for polymerase fidelity? These authors have presented the simplifying view that polymerase fidelity can be explained solely by differences in free energy between the chemical transition states for correct and incorrect nucleotide incorporation, avoiding the need to invoke more complex schemes involving induced fit or checkpoints. Since the overall fidelity of dNTP selection (the competition between correct and incorrect incoming nucleotides) is determined by comparing the free energy barriers between the ground state reactants and the highest-energy transition state on the respective reaction pathways for correct and incorrect dNTP incorporation (*16*), it follows that the scenario described by Showalter and Tsai requires that phosphoryl transfer be rate-limiting under both circumstances. However, for two of the most intensively studied DNA polymerases (Klenow fragment and T7 DNA pol), the available kinetic evidence has been interpreted as showing that a noncovalent step preceding phosphoryl transfer (step 3 in Scheme 1) is rate-limiting for correct dNTP incorporation, while phosphoryl transfer itself (step 4) is rate-limiting for an incorrectly paired incoming dNTP (*17*−*20*). Therefore, we should first examine critically the evidence for a rate-limiting noncovalent prechemistry step in the DNA polymerase reaction pathway.

[1] Abbreviations: Pol β, DNA polymerase β; T7 DNA pol, T7 DNA polymerase; T4 DNA pol, T4 DNA polymerase; HIV-RT, human immunodeficiency virus reverse transcriptase; Pol η, DNA polymerase η; Dpo4, *Sulfolobus solfataricus* DNA polymerase IV.

Rate-Limiting Steps in the DNA Polymerase Reaction Pathway

Although the sulfur elemental effect (the decrease in rate when an α-thio-dNTP is substituted for the normal all-oxygen nucleotide) has frequently been used as a diagnostic for whether the chemical step of the polymerase reaction is rate-limiting, it is now widely recognized that this is not a reliable criterion. From the work of Herschlag (*21*), the predicted elemental effect is relatively modest (∼4−10-fold) even if chemistry is completely rate-limiting, making it hard to interpret an elemental effect of, say, 2- or 3-fold. At the opposite extreme, elemental effects substantially above 10-fold have been observed under particular reaction conditions in some DNA polymerase systems (*20*, *22*). These large elemental effects are most probably steric in origin and consequently are no longer exclusively a diagnostic for the chemical step of the reaction. For polymerases such as Pol β, for which the evidence for a slow step preceding phosphoryl transfer is based largely on elemental effect arguments and analogy with other systems[2] (*24*, *25*), the assignment of this step in the reaction pathway should be regarded as provisional. For a few DNA polymerases (Klenow fragment, T7 DNA pol, HIV-RT, and two Y-family polymerases, yeast Pol η and archaeal Dpo4), however, there is much more persuasive evidence in favor of a slow noncovalent step preceding chemistry in the addition of a correctly paired dNTP (Table 1). In these polymerases, the lower yield from pulse−quench versus pulse−chase protocols indicates the presence of a dNTP-bound species that can form product instead of dissociating and equilibrating with the pool of free dNTP (*17*, *19*, *26*−*28*). The presence of a measurable quantity of such a dNTP-bound species, and its relatively facile conversion to product, require slow steps immediately preceding and following the chemical step in

[2] Deductions based on analogy should be viewed with caution in the case of Pol β because, on structural grounds, it is not a member of the polymerase superfamily (*23*).

the polymerase reaction pathway (steps 3 and 5 of Scheme 1).

From the point of view of evolution, there may be a considerable advantage to a polymerase that has overoptimized the rate of the chemistry step relative to the flanking steps (k_3 and k_5) in the turnover cycle. Polymerases catalyze a complex reaction, and if the enzyme is subject to pressures that result in changes in amino acid sequence, the fast rate of the chemical step may serve to buffer any effect such mutations may have on the rate of this step in the incorporation reaction cycle. As we will note later, the overoptimization of this step sets up fidelity checkpoints.

Data for T4 DNA pol argue that not all DNA polymerases may conform to this scheme. Correct dNTP insertion by T4 DNA pol gave the same product yield when quenched with acid or with EDTA, the latter serving as a surrogate for the pulse−chase protocol since it has been shown in other systems to allow conversion of a dNTP-bound intermediate to product (*29*). There are two possible interpretations of this result. The first is that, for T4 DNA pol, phosphoryl transfer is rate-limiting in the forward direction, even with a correct dNTP, and therefore any dNTP-containing complexes are in rapid equilibrium with the pool of unbound dNTP. Alternatively, the step following phosphoryl transfer may be fast so that an E*·DNA·dNTP species (in equilibrium with E*·DNA_{+1}·PP_i) cannot accumulate. For pulse−quench and pulse−chase protocols to show nonequivalence in yields, it is necessary that the chemical step of phosphoryl transfer be flanked by two steps (steps 3 and 5) whose rates are equivalent or slower than that of step 4. It seems likely that the requirement for a slow step 5 may not be met in the T4 DNA pol reaction pathway. As discussed below, step 5 provides a time window for editing a mismatch; given the high activity of the T4 3′−5′ exonuclease, the corresponding step 5 need not be slow. One can, for the time being, place DNA polymerases into two groups: those that undergo a rate-limiting noncovalent change before phosphoryl transfer during incorporation of a correctly paired dNTP (Klenow fragment, T7 DNA pol, HIV-RT, Pol η, Dpo4, and probably T4 DNA pol) and those for which there is insufficient evidence to make an assignment (Pol β and many others). Moreover, for those DNA polymerases that function *in vivo* as part of a larger complex, accessory proteins may also influence the rates of individual steps of the reaction.

Deductions about the nature of the rate-limiting step during misincorporation are based almost entirely on elemental effect data (summarized in Table 1), leading to the conclusion that phosphoryl transfer is rate-limiting for Klenow fragment, T7 DNA pol, Pol β, and Dpo4, but not for T4 DNA pol, HIV-RT, and Pol η (*18*, *20*, *24*, *27*, *28*, *30*, *31*). For the reasons already discussed, interpretations of elemental effect data are subject to many caveats so these assignments should be viewed with caution. For Klenow fragment misincorporation, identical product yields were obtained with acid and EDTA quench protocols, arguing in favor of rate-limiting phosphoryl transfer (*18*). However, the interpretation of this experiment is not without a caveat because of the slowness of the misincorporation reaction; even if phosphoryl transfer is not rate-limiting, it might not be fast enough to compete with the EDTA quench process (presumably the loss of metal ions from the catalytically poised complex), in which case no conversion of intermediate to product would be observed. Thus, even for Klenow fragment, the assignment of the rate-limiting step in misincorporation remains somewhat tenuous in the absence of methods that allow the separate detection of phosphoryl transfer and the preceding noncovalent change.

Do DNA Polymerases Achieve Maximum Fidelity?

As summarized in Table 1, the available kinetic evidence does not reveal a consistent pattern regarding which step in the polymerase reaction pathway is rate-limiting, particularly for misincorporation. In situations where different steps appear to be rate-limiting for correct and incorrect dNTP insertion (e.g., Klenow fragment or T7 DNA pol), this has the paradoxical effect of diminishing the specificity of the polymerase reaction below that which could be attained if the same step were rate-limiting in both situations. Consider the case of Klenow fragment, in which the available evidence (see above) indicates that step 3 of Scheme 1 is rate-limiting for correct dNTP incorporation, whereas step 4 (chemistry) is rate-limiting for misincorporation. Because step 4 is faster than step 3 for correct dNTP incorporation, it follows that the difference between the step 4 rates for correct and incorrect insertions will be greater than the difference between the rates of step 4 for misinsertion and step 3 for correct insertion (see Figure 1A). While the caveats regarding the identification of the rate-limiting steps for these reactions have been noted, this apparent paradox suggests to us that the polymerase reaction may be subject to additional constraints beyond merely the requirement to extract the maximum specificity from the process.

Although not illustrated in Figure 1, the steps following phosphoryl transfer also contribute to fidelity. For those polymerases possessing a 3′−5′ exonuclease function, the slow step 5 following step 4 increases the probability of editing the misincorporated base by partitioning [E*·DNA_{+1}·PP_i] to the exonuclease site (*32*). Although misincorporation slows step 4, it likewise appears to effectively slow step 5, so the probability of editing remains high (*18*). However, editing at this intermediate has not been demonstrated unequivocally and differentiated from classical proofreading, which acts later on [E·DNA_{+1}]. The efficiency of classical proofreading directly reflects the exonuclease activity, and in this context, it is noteworthy that the T4 3′−5′ exonuclease is $\approx 10^3$-fold more active than that of Klenow fragment (*18*, *29*) and so would be able to compete with corresponding faster steps following step 5.

The Identity of Step 3

For the majority of the DNA polymerases listed in Table 1, the rate of correct dNTP insertion is limited not by chemistry but by a noncovalent process that precedes chemistry (step 3 in Scheme 1). What, then, is this noncovalent step? The prevailing hypothesis, widely quoted in the literature, is that the kinetically defined "conformational change" corresponds to the open-to-closed conformational change inferred from structural studies (pathway A in Figure 2). Though attractive, this hypothesis is unsupported by experimental data. Stopped-flow fluorescence studies of several DNA polymerases are equally consistent with an alternative hypothesis that the closing of the fingers subdomain is a rapid early step that occurs immediately after dNTP binding. In these experiments, a 2-aminopurine reporter at

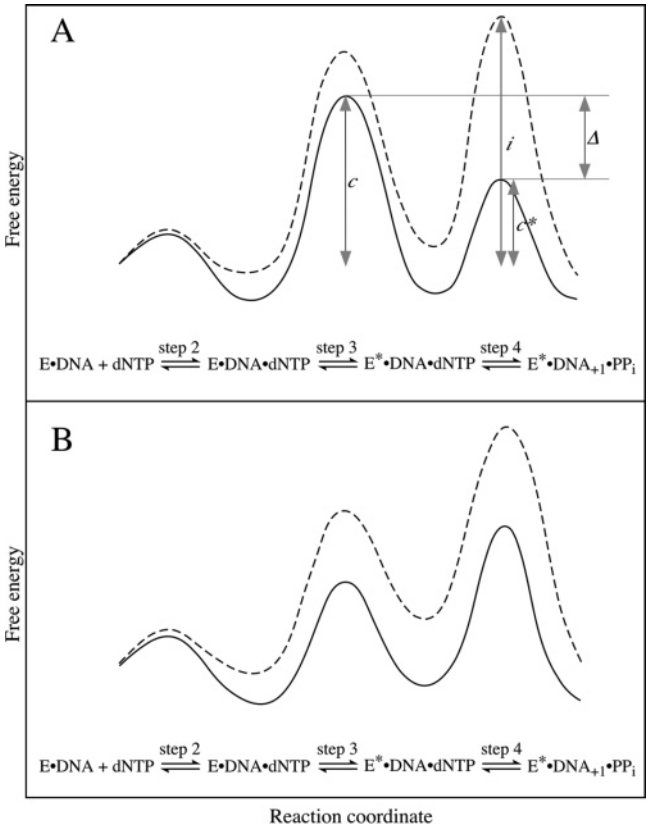


FIGURE 1: Schematic illustration of free energy profiles for the polymerase reaction. (A) Comparison of correct (—) and incorrect (- - -) dNTP incorporation by a polymerase such as Klenow fragment, for which step 3 is rate-limiting (in single turnover) for correct insertion and step 4 is rate-limiting for misinsertion. Specificity for the correct nucleotide is derived from the difference in heights of the largest energy barrier in each pathway: c (correct) and i (incorrect). If step 3 were much faster, making step 4 rate-limiting under both circumstances, c would be replaced by c*, and the specificity would be greater by an amount indicated as Δ. (B) Hypothetical free energy profiles for a polymerase in which step 4 is rate-limiting for both correct and incorrect dNTP incorporation. Intermediate steps on the misincorporation pathway (- - -) have a higher free energies than those on the correct incorporation pathway (—), and are therefore possible kinetic checkpoints. Note that the slow step 5 is not included in these profiles, although it, too, can influence fidelity, as discussed in the text.

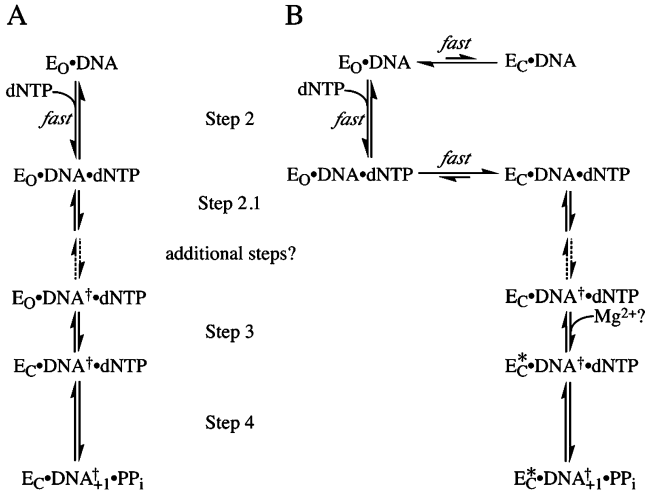


FIGURE 2: Hypothetical reaction pathways for the DNA polymerase reaction up to the phosphoryl transfer step. The pathways differ in whether the open (E_O)-to-closed (E_C) transition is slow (A) or fast (B). Formation of $DNA^\dagger$ in step 2.1 represents the conformational rearrangement that is detected with 2-aminopurine 5′ to the templating base. In pathway B, transformation of E to E* represents the rate-limiting noncovalent transition (step 3) whose identity is not established.

the templating position gave a fluorescence change that was substantially faster than k_3, ≈300 s^{-1} for pol β and >1000 s^{-1} for Klenow fragment and T4 DNA pol (*33*−*36*). Further, if the conversion between open and closed conformations were fast, the polymerase domain could be in rapid equilibrium between open and closed forms, even in the absence of dNTP. Binding of dNTP would stabilize the closed conformation and displace the equilibrium from, for example, 90% in the open form to 90% in the closed form (pathway B in Figure 2). In pathway B, the rate-limiting step 3 would be assigned to some other noncovalent transition. The two alternative pathways in Figure 2 have been expanded beyond the minimal pathway (Scheme 1) to illustrate the likelihood of additional rapid steps before the rate-limiting step, consistent with the observations in stopped-flow fluorescence studies with Klenow fragment (*35*). These hypothetical unassigned steps would correspond to rearrangements of the open complex in pathway A and the closed complex in pathway B. Included in these steps is the previously identified step 2.1, detectable as a fluorescence increase with a 2-aminopurine reporter 5′ to the templating base.

Currently, the evidence to distinguish between pathways A and B is entirely circumstantial. It is tempting to assume that the dramatic structural rearrangements associated with closing of the fingers and forming a binding pocket for the nascent base pair would result in a large fluorescence change. Following this reasoning, the stopped-flow data using 2-aminopurine at the templating position would argue in favor of pathway B because step 3 is associated with little or no fluorescence change, whereas the early rapid step causes a substantial fluorescence decrease (*35*). Obviously, alternative interpretations of the data cannot be ruled out. A more persuasive line of evidence concerns dNTP binding affinity, which is affected both by complementarity to the templating base and by particular side chains on the fingers subdomain (*18*, *25*, *37*−*39*); thus, an incoming dNTP must be able to sample all of these interactions as part of the binding process. This requirement would be satisfied by pathway B, in which the incoming dNTP has access to the closed conformation of the polymerase domain within the binding equilibrium; conversely, in pathway A, the incoming dNTP is unable to sample the closed conformation because of the intervening slow step 3 that follows the establishment of the dNTP binding equilibrium. Therefore, if pathway A is the correct pathway, some other means of checking the incoming dNTP against the templating base must exist. Intriguingly, recent structural studies of T7 RNA polymerase, a close structural homologue of the A-family DNA polymerases (Klenow fragment, T7 DNA pol, etc.), suggest a means of checking complementarity with the polymerase domain in the open conformation (*40*). If an analogous checking mechanism were to exist in DNA polymerases, it would allow the dNTP binding constant to respond to the nature of the templating base even if the closing of the fingers were a slow step that was not part of the binding equilibrium. Ultimately, the determination of whether the open-to-closed transition of the polymerase domain is an early rapid step

or the rate-limiting prechemistry step will require direct observation of the rate of this conformational change.

If the open-to-closed transition is rapid, as in pathway B (Figure 2), other possibilities that have been suggested for the rate-limiting step 3 include the binding of the second metal ion at the polymerase active site, rotation of one or more important side chains, or some other conformational rearrangement at the active site (*25*, *34*, *35*, *41*). The extent to which the slow step 3 is a discriminator between correct and incorrect base pairs may vary from polymerase to polymerase. In the Klenow fragment system, it was possible to simulate the observed kinetics of correct and incorrect incorporations without requiring the rate of step 3 to be different in the two cases (*18*). The opposite conclusion was made in the T7 DNA pol system, but this was based on questionable deductions from elemental effect data (*20*). The observed elemental effects of 20−40-fold for misincorporation were interpreted as indicating that phosphoryl transfer was only partially rate-limiting. If, on the other hand, one concluded that chemistry was entirely rate-limiting, the preceding step would be kinetically invisible and its rate would be less well constrained, resembling the Klenow fragment situation. Thus, although there is good evidence for the existence of a slow step 3 in Klenow fragment and T7 DNA pol, the available data cannot tell us whether the rate of this step is influenced by the matching of the incoming dNTP to the templating base. Conversely, for those polymerases for which step 3 appears to be rate-limiting in misincorporation (T4 DNA pol, HIV-RT, and Pol η), this step must discriminate between correct and incorrect nascent base pairs and could be a major contributor to the fidelity of these enzymes.

Existence of Kinetic Checkpoints

The overall rate of the polymerase reaction is determined by the difference in free energy between the ground state reactants and the highest energy barrier on the reaction pathway. Polymerase fidelity is then determined by the relative heights of the free energy barriers to be surmounted in correct and incorrect dNTP incorporations. In a complex enzymatic reaction, involving the association of the enzyme with more than a single reactant, it is unlikely that the highest energy barrier will be approached in a single step from the ground state reactants. Instead, there will be one or more intermediate species along the way, and these can serve as kinetic checkpoints. The presence of these checkpoints will not change the overall fidelity of the reaction, but they define the pathway by which that fidelity is realized. In the polymerase reaction, the intermediate steps or checkpoints will allow rejection of inappropriately paired dNTPs before the polymerase attempts phosphodiester bond formation on such substrates. Consider the free energy profile shown in Figure 1B for a hypothetical DNA polymerase in which phosphodiester bond formation is rate-limiting for both correct and incorrect dNTP incorporations; this is the scenario favored by Showalter and Tsai (*15*). Because of the higher energy barrier for misincorporation, it follows that the intermediates along the misincorporation pathway will have higher energies than the corresponding species in correct incorporation, resulting in slower forward reaction rates, faster reverse reaction rates, or some combination of these attributes. Consequently, the misincorporation intermediates will be more likely to decay back to starting materials. Although checkpoints are not absolutely necessary to achieve the fidelity inherent to the polymerase reaction, we argue that their existence is a natural consequence of the form of the energy profile. Moreover, such checkpoints define operationally the mechanism by which DNA polymerase specificity is manifested. Early checkpoints may serve a useful purpose by allowing discrimination between correct and incorrect nucleotides to be expressed early in the reaction pathway, benefiting the overall throughput of the reaction by reducing the time spent processing inappropriate substrates. Checkpoints after phosphoryl transfer influence the selectivity of the 3′−5′ exonuclease editing function.

While the structural similarities between DNA polymerases and their substrate complexes (*42*) may imply conservation of the reaction pathway and the intermediates or checkpoints on it, the available evidence suggests that the relative importance of the various checkpoints may not be strictly conserved. For example, the extent to which the dNTP binding step serves as a discriminator is highly variable. Two closely related polymerases, Klenow fragment and T7 DNA pol, illustrate the extremes of behavior, with T7 DNA pol showing very strong discrimination in the ground state binding of correct and incorrect dNTPs, and Klenow fragment much less (*20*, *37*). Likewise, the differences in the relative rates of phosphoryl transfer and the preceding nonchemical step (Table 1) indicate variability in the energetics of the reaction pathway for different polymerases, perhaps related to their distinct functions *in vivo*.

Structural Descriptions

On the basis of the crystal structures of DNA polymerases and their complexes, and stopped-flow fluorescence data, we can hypothesize structures for species on the polymerase reaction pathway (see Figure 2). The initial encounter between a dNTP and the Pol−DNA binary complex is likely to involve the open conformation because of limited access to the dNTP binding pocket in the closed conformation, and will probably use contacts between positively charged side chains of the fingers subdomain and the triphosphate tail of the dNTP, as seen in polymerase−dNTP complexes in the absence of DNA (*43*, *44*). In pathway B, with the open conformation in rapid equilibrium with the closed conformation, the fingers would carry the dNTP into the active site where it would encounter the templating base. If the dNTP is complementary to the templating base, the closed conformation would be stabilized and would predominate in the equilibrium mixture. Conversely, a noncomplementary dNTP would not stabilize the closed conformation and would be more likely to dissociate from the predominantly open ternary complex, providing an important kinetic checkpoint. The closed ternary complex with a correctly paired dNTP would then undergo several noncovalent transformations, which include a rapid rearrangement resulting in changes in the environment of the 5′ neighbor of the templating base (step 2.1), and the slow step (perhaps binding of a metal ion) that is rate-limiting for the majority of DNA polymerases. The end result of these noncovalent steps would be an active site poised for catalysis of phosphoryl transfer, to extend the primer strand by one nucleotide.

If, on the other hand, the closing of the fingers is rate-limiting (pathway A), we postulate one or more conforma-

tional rearrangements that allow the dNTP to "preview" the templating base within the open ternary complex. These hypothetical steps might correspond to the early fluorescence changes that have been observed using 2-aminopurine reporters. Complementarity between the dNTP and the template would stabilize the dNTP binding interaction, favoring the forward reaction, whereas a lack of complementarity would favor dissociation. The open ternary complex, perhaps in a conformation in which the template−dNTP interaction is partially formed, would then undergo the rate-limiting conversion to the closed complex, followed by phosphoryl transfer. Thus, in either pathway of Figure 2, the dNTP binding process is a potential kinetic checkpoint whose discrimination efficiency will be dictated by the structure of the polymerase.

How does a mispaired dNTP impact either of the two proposed reaction pathways? First, as already discussed, the dNTP binding equilibrium will be destabilized, favoring dissociation and resampling of the dNTP pool. Second, if the mispaired ternary complex proceeds along the reaction pathway, it is likely to form intermediates whose geometry and/or energetics are inappropriate for phosphoryl transfer. Thus, in pathway B, a noncomplementary dNTP could give a ternary complex that is predominantly in the open form. It seems unlikely that the polymerase could carry out phosphoryl transfer directly from the open conformation, so the likely scenario is that the reaction would proceed through a sparsely populated closed ternary complex that may differ significantly from the active site geometry of the analogous complex in correct dNTP insertion. On the other hand, if pathway A applies, steric problems with accommodating a nascent mispair in the closed complex may be felt in the transition state for step 3, resulting in a very high activation energy barrier. If conformational rearrangements are required in preparation for step 3, it is also possible that these may be highly unfavorable when the incoming dNTP is mispaired.

It is probably unrealistic to envisage a single generic misinsertion pathway. The distinct geometries of nascent mispairs would be expected, naively, to generate three subsets of responses: to nascent wobble mispairs (correct size but distorted geometry), to purine-purine mispairs (too large), and to pyrimidine-pyrimidine mispairs (too small). Even within these categories, it is clear from biochemical experiments that some mispairs are more detrimental to polymerase incorporation than others (*37*, *45*, *46*). Our preliminary fluorescence data for misinsertion reactions showed a variety of responses depending on the identity of the mispair (*35*), but, importantly, all were different from the correct dNTP insertion, particularly in the absence of a signal corresponding to changes involving the 5′ neighbor of the templating base (step 2.1). Such observations support the contention that nascent mispairs do not necessarily achieve the same active site geometry as correct pairs, so one could envisage the polymerase reaction as a group of parallel but subtly different reaction pathways corresponding to correct dNTP insertion and to subsets of misinsertion reactions.

The idea of nonidentical reaction pathways for correct and incorrect dNTP incorporations resembles the generalized induced-fit model described by Post and Ray (*47*). According to this model, the enzyme would accommodate both good (correct pair) and poor (mispair) substrates in conformationally distinct manners, but the complexes with the poor substrate are such that the reaction rate would be compromised and/or intermediates would have unfavorable energetics. This formulation contrasts with the more traditional view of induced fit (*16*), in which the identical transition state geometry is imposed on both good and poor substrates and selectivity is derived from the differing abilities of substrates to conform to this geometry. As Post and Ray point out (*47*), conformational flexibility in enzymes is widespread, but does have the paradoxical effect of diminishing the specificity below that which can be obtained with a (hypothetical) rigid active site. In the polymerase system, conformational flexibility may have the advantage of providing intermediate states that can serve as kinetic checkpoints, and may also allow alternative reaction pathways that are required under special circumstances, for example, when a polymerase encounters a damaged template.

The recently published structures of mismatched primer termini at a DNA polymerase active site provide a tantalizing view of possible intermediates following phosphoryl transfer on the misincorporation pathway (*48*). Some mispairs are observed in a pretranslocation and some in a posttranslocation position, suggesting that an important feature of the reaction pathway is the residence time of the newly synthesized base pair in the polymerase active site before translocation. This could allow reversal of the reaction by pyrophosphorolysis, as has been reported for chain-terminating nucleotide inhibitors of HIV-RT (*49*), or could influence the interplay between polymerase and 3′−5′ exonuclease activity for those DNA polymerases with proofreading activity (*32*).

Future Directions

Further understanding of the mechanisms underlying polymerase fidelity would be helped immensely by a more detailed structural characterization of the species on the misincorporation reaction pathway. However, the probable instability of many of these complexes may handicap crystallographic approaches. For example, if polymerase structures with nascent mispairs in the active site are likely to adopt an open conformation with a loosely bound dNTP, then these complexes will be difficult to capture by crystallization. Given the multiple conformational interconversions that appear to take place on the polymerase reaction pathway, valuable information should be obtained using spectroscopic techniques that probe the conformational dynamics of the polymerase and its substrates, which is being attempted in some systems (*50*, *51*).

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