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REGULAR ARTICLE

Perspective: pre-chemistry conformational changes in DNA polymerase mechanisms

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Abstract In recent papers, there has been a lively exchange concerning theories for enzyme catalysis, especially the role of protein dynamics/pre-chemistry conformational changes in the catalytic cycle of enzymes. Of particular interest is the notion that substrate-induced conformational changes that assemble the polymerase active site prior to chemistry are required for DNA synthesis and impact fidelity (i.e., substrate specificity). High-resolution crystal structures of DNA polymerase β representing intermediates of substrate complexes prior to the chemical step are available. These structures indicate that conformational adjustments in both the protein and substrates must occur to achieve the requisite geometry of the reactive participants for catalysis. We discuss computational and kinetic methods to examine possible conformational change pathways that lead from the observed crystal structure intermediates to the final structures poised for chemistry. The results, as well as kinetic data from site-directed mutagenesis studies, are consistent with models requiring pre-chemistry conformational adjustments in order to achieve high fidelity DNA synthesis. 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.

Keywords Enzyme catalysis · Intrinsic protein dynamics · Pre-chemistry conformational adjustments · Nucleotidyl transfer · DNA polymerase β · Catalytic cycle chemical step

Editor's Note: This paper and papers by Mulholland AJ, Roitberg AE, Tuñón I (doi:10.1007/s00214-012-1286-8) and Ram Prasad B, Kamerlin SCL, Florián J, Warshel A (doi:10.1007/s00214-012-1288-6) document and discuss contrasting outlooks on the questions of pre-chemistry 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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1 Introduction

Recent papers have provided a lively exchange concerning theories for enzyme catalysis, specifically the role of intrinsic dynamics in the catalytic cycles as deduced from molecular simulations (e.g., [1–8]). The issues involve the functional significance of these dynamics, the relation of conformational to chemical transitions, and the utility of theories that emerge from atomistic simulations concerning motions and associated energy landscapes. Various papers have pointed out issues of semantics in these discussions concerning what is dynamics: correlated conformational changes that occur throughout the enzyme's pathway and

act to facilitate the chemical reaction on one hand, versus rearrangements that have a direct link to the transition state in the chemical reaction on the other [6]. It has also been generally concluded in many of 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 [4].

DNA polymerases are multi-domain enzymes with a polymerase domain and accessory domains. The polymerase domain is responsible for the nucleotidyl transferase reaction and is usually composed of three subdomains. DNA polymerase (pol) β is the simplest eukaryotic DNA polymerase and has been well characterized structurally [9]. A key structural observation that has been made for several DNA polymerases is that a subdomain closes around the nascent base pair upon binding of the incoming correct nucleoside triphosphate, to trap it between the growing DNA duplex and the polymerase [10, 11]. The transition from the open polymerase/DNA binary complex to the closed ternary polymerase/DNA/dNTP complex is accompanied by conformational adjustments in key protein side chains, DNA nucleotides, and the incoming nucleotide. In addition, the binding of two metal cofactors, Mg^{2+}, is required for chemistry and is known to influence local conformations and subdomain motions [12, 13].

Although it is generally believed that chemistry is the rate-limiting step during DNA synthesis for DNA polymerases, the lack of a straightforward assay to assess whether polymerase and/or substrate conformational changes limit DNA synthesis in specific cases (e.g., altered enzymes or substrates) dictates that activity measurements should be interpreted cautiously. In the specific case of pol β, the role of the assembly of enzyme side chains, substrates, and metals necessary to achieve optimal geometry for chemistry has been recently discussed. Warshel and Prasad argue that conformational adjustments preceding the pol β chemical step are inconsequential to understanding catalysis or fidelity [14]. We present an alternate viewpoint: such adjustments have a role in catalysis since they are a pre-requisite for DNA synthesis. In this perspective, we elaborate on this point and review challenges to integrating results from kinetic, structural and computational approaches toward understanding polymerase catalysis.

2 Induced-fit and pre-chemistry conformational changes

As already recently argued in the literature [3–7], an understanding of enzyme-catalyzed reactions requires knowledge of many steps, conformational and chemical, with the later being just one part of the big picture [5]. There is increasing support for pre-chemistry conformational changes as an important component in the overall catalytic cycle for pol β. Many of the pre-chemistry atomic adjustments have been revealed through X-ray crystallography [12, 15], NMR spectroscopy [13], and fluorescence spectroscopy [16] and have also been studied using computational approaches (e.g., [17–21]). The combined picture of the energy landscape is needed to link atomistic details with results from various experimental methods and pursue enzyme design applications.

The induced-fit hypothesis [22] argues that the alignment of catalytic groups be optimized for high activity and that substrate binding provides energy to overcome the unfavorable conformation. As noted above, crystallographic structures of DNA polymerases in various liganded states indicate that subdomain re-positioning is thought to be an essential step to ready the system for the chemically competent state. Originally, these subdomain motions were thought to be rigid, but computer simulations revealed an unexpected complexity in subtle side-chain motions that evolve the system into the state ready for chemistry [23, 24]. This work also suggested that it is these side-chain motions that are rate limiting in the *conformational closing pathway* rather than the overall subdomain closing motion. Such comparative studies for matched (correct) and mismatched (incorrect) incoming nucleotides (e.g., [17, 25–28]) thus led to a better understanding of the role of specific enzyme residues as well as ions in organizing the active site. Our suggestion of pre-chemistry conformational changes in this connection [23] is a specific application of the induced-fit hypothesis. For this discussion, the terms dynamics or pre-chemistry conformational changes refer to substrate or enzyme atomic rearrangements that have catalytic consequences, that is, events that impact phosphoryl transfer chemistry.

There have been many studies exploring alternative or conjunctive mechanisms to induced-fit, namely conformational selection [29–33]. Though the difference is subtle and refers to whether the enzyme has the intrinsic capacity to undergo the requisite conformational changes (which are merely favored when substrate binds via a shift in equilibrium in the conformational energy landscape) or whether the substrate actually triggers these changes, dynamics studies suggest that a hybrid mechanism is operative for polymerases of the X-family [34]. In fact, follow-up work suggested that while subdomain motions appear intrinsic to the DNA polymerase, the subtle side-chain motions have higher activation energy barriers and their dynamics and favored states are largely determined by the binding of the substrate [35].

Figure 1 shows this behavior of the potential mean force for the pol β closing pathway prior to chemistry, as

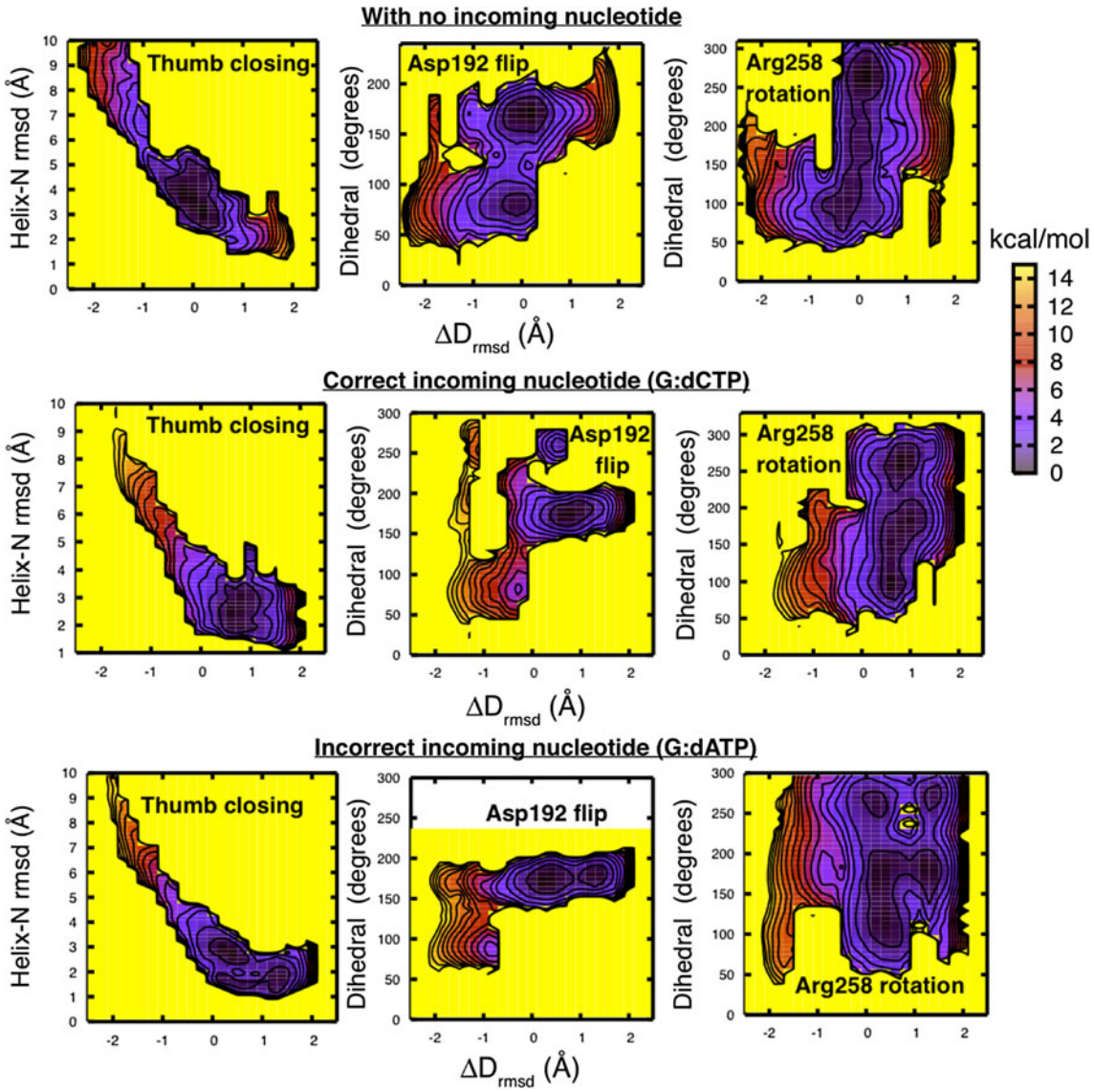

Fig. 1 Quantitative characterization of the open/closed transitions in pol β/DNA complex without dNTP and in the presence of correct (dCTP) and incorrect (dATP) incoming nucleotide (*top to bottom*) [35]. Shown are two-dimensional free energy surfaces as a function of the ΔD_{rmsd} progress variable for three order parameters: α-Helix N rmsd with respect to the closed crystal state, and dihedral angles of key active-site residues, Asp192 and Arg258. ΔD_{rmsd} is the difference in rmsd value of the instantaneous structure from the reference open and closed states: negative values correspond to the more open-like state while positive values correspond to closed-like state. Near the open and closed states, the values of the order parameters range as follows: α-helix N rmsd, from 6 to 1.5 Å; Asp192 flip, from 90° to 180°; and Arg258 rotation, from 100° to 260°. These order parameters characterize the closing transition of pol β and were previously identified as transition states along the pathway [24]. Here, the free energy profiles were obtained by performing umbrella sampling simulations on optimized NEB paths. In umbrella sampling simulations, weak harmonic restraints were placed along the ΔD_{rmsd} progress variable. The data were unbiased using WHAM to obtain free energy surfaces

computed using the nudged elastic band method [29] for three order parameters (subdomain motion, Phe272 ring flipping, and rotation of Arg258) in three cases where the templating (coding) base is guanine: 1) without the correct dCTP substrate (top), 2) with correct dCTP (middle), and 3) with incorrect dATP (bottom). We see that while the subdomain motion is essentially a barrier-free motion in agreement with prior studies [24], substrate binding modulates the distribution of equilibrium enzyme conformations of key side-chain conformations as needed for the chemical reaction. These data are in agreement with single-molecule FRET studies of the Klenow fragment of Pol I showing a high degree of conformational flexibility in the unliganded state and occupancy of distinct conformational states other than the open and closed conformations in the presence of an incorrect substrate [36]. These results are

also in line with similar single-molecule studies of HIV-1 reverse transcriptase that suggest conformational heterogeneity of the polymerase/DNA complex depending on the binding register of the DNA substrate [37]. The collective data thus indicate the applicability of the conformational selection model: substrate binding to a specific polymerase conformation *shifts* the energy landscape to favor one state over another rather than triggering a transition per se. The studies are applicable to many other enzymes [38–40] and further underscore the range and relevance of enzyme dynamics for understanding their complete mechanism.

3 Influence of pre-chemistry conformational changes on enzyme efficiency and fidelity

Although protein and substrate conformational adjustments are required to optimize the active-site architecture for efficient chemistry, the role of these adjustments in substrate specificity (fidelity) is controversial (see [41] for discussion). In general, DNA polymerase fidelity is the ratio of insertion efficiencies for right and wrong incoming nucleotides. High fidelity DNA polymerases have high insertion efficiencies for the correct nucleotide, whereas the insertion efficiency for incorrect nucleotides is much lower and generally similar for all DNA polymerases irrespective of their fidelity [42].

Site-directed mutagenesis of DNA polymerases has demonstrated that some of the altered enzymes have decreased fidelity. Accordingly, correct and/or incorrect nucleotide insertion efficiencies must be *differentially* affected with the mutant enzyme, suggesting that the altered side chain plays a unique role(s) for correct and incorrect insertion. For example, 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 (i.e., open enzyme form) (Fig. 2) [43]. Replacing this arginine side chain with alanine 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 [42, 44]. Thus, Arg283 appears to play an essential role in pre-chemistry events for correct, but not incorrect, nucleotide insertion.

Likewise, Glu295 has been implicated in providing key contacts as the polymerase transitions from an open to closed complex (Fig. 2) [13]. It has also been shown that the enzyme is catalytically compromised upon lysine substitution [45, 46]. A crystal structure of a binary DNA complex of the E295K mutant indicates an intermediate conformation between open and closed forms [47]. Further computational work [47] supports a very high-energy barrier for the closing conformational pathway of this enzyme due to electrostatic and steric factors (Fig. 3). In fact, the high-energy barrier comes not from subdomain closing but from the rotation of Arg258 that must release Asp192 to coordinate active-site metals. Indeed, NMR studies are consistent with the idea that the E295K mutant is unable to achieve a closed 'activated' complex in the presence of a correct nucleotide/Mg^{2+} [13]. Thus, this example underscores the need to understand conformational as well as chemical events: mutants may exist with conformational transitions that are not only important but possibly rate limiting.

Although high-resolution structures of DNA polymerases from several families in various liganded states have provided valuable insights into the structure and mechanism of DNA polymerases, correlating these structures with observed kinetic behavior is not straightforward. These structures reveal that the protein and substrates must undergo structural changes to attain the geometry necessary for chemistry. Appropriately, these conformational changes should align catalytic groups for efficient correct nucleotide insertion and misalign catalytic atoms for inefficient incorrect nucleotide misinsertion. Indeed, structures of pol β with active-site mismatches reveal that subdomain closure misaligns key catalytic atoms [19, 48].

For nucleotide binding to a polymerase/DNA complex, a simple representation of a two-step nucleotide-binding model proposed by Johnson and Benkovic [49] is illustrated in Fig. 4. Historically, indirect kinetic evidence suggested that a non-chemical step limited the overall rate (i.e., k_2 and k_{-2} were slow relative to k_3, Fig. 4); in other words, a conformational change believed to be the open (ES) to closed (*ES) polymerase transition was rate limiting for correct nucleotide insertion. More recently, the rate of the open to closed enzyme transition [50–53] or template conformational adjustments [16, 54] has been experimentally determined to be faster than chemistry. This indicated that the chemical step was the slowest step for the forward reaction. Tsai and Johnson point out, however, the efficiency (k_{cat}/K_m) of correct nucleotide insertion is independent of the chemical step (k_3) [41, 52] even when it is slow. For correct insertion, efficiency is approximately K_1k_2 where $K_1 = k_1/k_{-1}$. Critically, catalytic efficiency is linked to the reversal of the polymerase conformational change (k_{-2}; where $k_{-2,\text{incorrect}} \gg k_{-2,\text{correct}}$; i.e., $K_2 = k_2/k_{-2} =$ [*ES]/[ES] $\ll 1$). Thus, the concentration of *ES accumulates for correct insertion, but not for incorrect insertion (i.e., [ES] is high). The significance of this simple result is that chemistry, even when the slowest step, cannot be directly measured in all situations since the measured rate is k_3[*ES] and [*ES] will depend on the magnitude of several rate constants including k_{-2}. The conformational change forming *ES insulates the correct nucleotide from rapidly dissociating from the ES complex (nucleotide binding is in

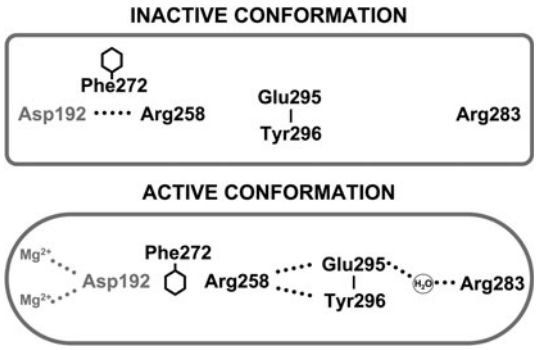

Fig. 2 Altered side-chain interactions accompanying subdomain closure of pol β. In the open inactive conformation, Arg283 does not interact with other key residues, but in the closed active conformation, it interacts with the templating (coding) base, the upstream template nucleotide (not illustrated), and with Glu295 (indirectly). Thus, the position of the subdomain can be structurally transmitted to the active site through a series of interactions involving Arg283 permitting Asp192 to coordinate both active-site Mg^{2+} ions. This is also accompanied by altered interactions of Glu295/Tyr296 with Arg258 in the open (inactive) and closed (active) forms. Phe272 is postulated to transiently interfere with interactions between Asp192 and Arg258 permitting an interaction with Glu295/Tyr296. Residues of the subdomain that closes are indicated in boldface text. This figure was reproduced from Ref. [13] with permission

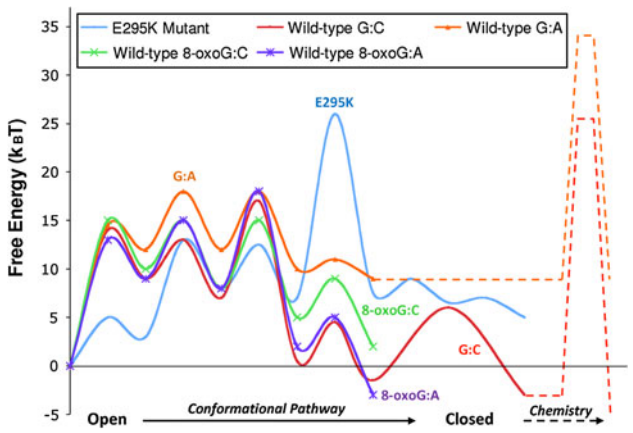

Fig. 3 Estimated conformational and chemical pathways and/or barrier for four pol β systems as collected from several works: wild-type system with correct and incorrect incoming bases [17, 19, 20]; wild-type system with the 8-oxoG DNA lesion [59]; and for E295K mutant [47]. All conformational barrier calculations employ transition path sampling, and the chemistry estimates are obtained from various groups using QM/MM and EVB techniques

rapid equilibrium; i.e., $k_{-1} \gg k_2$) by forming a complex (*ES) with a slower reverse rate constant (k_{-2}). In contrast, when k_{-2} is rapid, as in the case for an incorrect nucleotide, *ES does not accumulate and the wrong nucleotide can quickly dissociate from the ES complex. 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

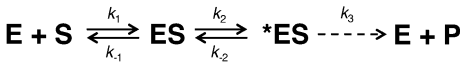

Fig. 4 Two-step substrate binding model. Enzyme (E) binds a substrate/ligand (S) to form an initial collision complex (ES). This complex undergoes a conformational change to form a productive complex (*ES) that can undergo chemistry to form products (P). For many DNA polymerases, the ES and *ES states would represent the open DNA binary and closed dNTP ternary complexes, respectively, observed crystallographically. Note that when chemistry (k_3) is rate limiting, the observed velocity will depend on the concentration of *ES. Observed changes in the rate of product formation can be due to changes in k_3 and/or changes in the concentration of *ES. Nucleotide binding to DNA polymerases is believed to utilize a two-step binding mechanism [41, 49, 52]. Accordingly, attempting to interpret or assign energy barriers to activity measurements is problematic, since the distribution of intermediate enzyme species is not known

rate cannot be directly related to chemistry or conformational changes.

This simple analysis applied to DNA polymerase only accounts for nucleotide binding and does not address other conformational adjustments in the substrates (i.e., DNA translocation) that also must occur. Additionally, pyrophosphorolysis (reverse of step 3 in Fig. 4, not shown) and post-chemistry conformational changes would alter the distribution of enzyme species thereby confounding the interpretation of experimentally determined rates. Kinetic characterization of the sensitivity of the mitochondrial DNA polymerase γ to nucleoside analogs has demonstrated that the reverse reaction and post-chemistry conformational changes can influence nucleotide insertion [55]. From this discussion, it is apparent that interpretations of activity measurements based solely on steady-state or transient-state kinetic approaches can be error prone, since they rely on the precise mechanism and magnitude of the intrinsic rate constants. A kinetic mechanism that defines the rate constants for each pertinent step should be consistent with results from both of these kinetic approaches and provide a deeper understanding of the distribution of intermediate enzyme complexes during catalytic cycling. The ambiguity in defining the distribution of enzyme complexes during catalytic cycling makes correlating computationally determined energy barriers to specific enzymatic steps a challenge. This challenge also extends to correlating high-resolution crystallographic structures to specific enzymatic intermediate species that have been postulated through kinetic characterization and the inferred events that must occur during transition between these species.

The observation that conformational changes are required for catalytic activation supports the concept of an induced-fit mechanism proposed by Koshland [22] and related mechanisms [29–33]. It has been pointed out that induced fit can alter enzyme specificity even when critical conformational changes are not rate limiting [56], such as when the transition states for correct and incorrect

incorporation are unique. Although an induced-fit model reduces enzyme specificity relative to a situation where a unique transition state exists, the reduced specificity represents an acceptable compromise for an enzyme such as a DNA polymerase, which must select a different/new substrate (DNA and dNTP) with each insertion event.

4 Concluding remarks

The complex internal motions and simulation of the entire configurational landscape are essential to understanding function as well as how enzymes achieve regulation and function with different substrates and through various protein associations. The commentary by Mulholland et al. [57] also underscores the importance of molecular simulation to make connections with experiment, to predict behavior of mutant systems and alternative substrate, and to connect the conformational to chemical pathways, as we have done for various polymerases in many different contexts of substrates, mutations, and other conditions. These combined pathways and conformations are utilized by DNA polymerases to enhance fidelity. Clearly, works that adopt these broader views and attempt to connect the many aspects of enzyme activity and function help contribute to the deciphering of complex enzyme machineries, of which polymerases are notable in both their versatility and fidelity. From an overview of catalytic efficiencies for divergent DNA polymerases, it is clear that high fidelity DNA polymerases are optimized for correct nucleotide insertion. These high fidelity enzymes exhibit subdomain, enzyme side chain, and substrate adjustments that contribute to optimizing the electrostatics and geometry of the active site [10]. Thus, substrate-induced conformational changes that assemble the polymerase active site prior to chemistry are required for DNA synthesis.

Crystallographic structures [12, 44, 47], spectroscopic NMR studies [13], and kinetic evidence [44, 46] highlight critical residues believed to play an important role in these pre-chemistry events for pol β. Although these pre-chemistry events are difficult to experimentally measure, computational approaches can provide models that bridge crystallographic structures and kinetic properties.

Computational methods are subject to the well-known approximations and imperfections of force fields and sampling [58], but perfection is not a pre-requisite for utility. Ultimately, the success of such predictions and interpretations can be tested experimentally. Work that attempts to merge a range of experimental knowledge and computational insights is valuable. Many computational studies by a variety of approaches have added further insights to the behavior of mutant enzymes by describing altered energetic and dynamic scenarios for alternate enzyme/substrate conformations, their effect on the overall enzymatic reaction, and associated feasible reaction pathways. Such cumulative data provide a framework to interpret a wide range of behavior observed with different polymerase systems across several DNA polymerase families and stimulate the design of further experiments. A view that focuses on the chemical barrier [14] may overlook important steps required to achieve a catalytically competent state and, accordingly, cannot always reliably predict the behavior of altered enzymes.

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