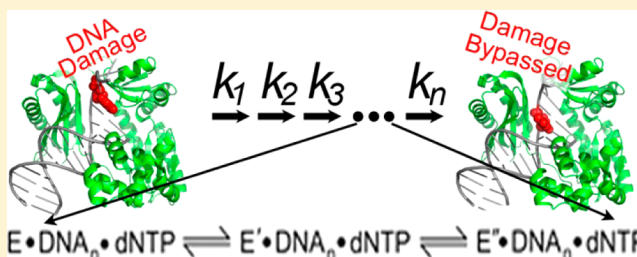


# Recent Insight into the Kinetic Mechanisms and Conformational Dynamics of Y-Family DNA Polymerases

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**ABSTRACT:** The kinetic mechanisms by which DNA polymerases catalyze DNA replication and repair have long been areas of active research. Recently discovered Y-family DNA polymerases catalyze the bypass of damaged DNA bases that would otherwise block replicative DNA polymerases and stall replication forks. Unlike DNA polymerases from the five other families, the Y-family DNA polymerases have flexible, solvent-accessible active sites that are able to tolerate various types of damaged template bases and allow for efficient lesion bypass. Their promiscuous active sites, however, also lead to fidelities that are much lower than those observed for other DNA polymerases and give rise to interesting mechanistic properties. Additionally, the Y-family DNA polymerases have several other unique structural features and undergo a set of conformational changes during substrate binding and catalysis different from those observed for replicative DNA polymerases. In recent years, pre-steady-state kinetic methods have been extensively employed to reveal a wealth of information about the catalytic properties of these fascinating noncanonical DNA polymerases. Here, we review many of the recent findings on the kinetic mechanisms of DNA polymerization with undamaged and damaged DNA substrates by the Y-family DNA polymerases, and the conformational dynamics employed by these error-prone enzymes during catalysis.



DNA polymerases perform a variety of critical functions involved in the replication, repair, and processing of genomic DNA,<sup>1</sup> and their kinetic mechanisms have long been of great interest. On the basis of phylogenetic analysis, six distinct DNA polymerase families have been identified: A–D, X, and Y. DNA polymerases from all families use a two-divalent metal ion mechanism for nucleotide incorporation with a common minimal kinetic pathway<sup>2–4</sup> and share a structurally conserved polymerase core architecture consisting of finger, thumb, and palm subdomains in a “right-hand” geometry.<sup>2,5–9</sup> Despite these similarities, DNA polymerases differ greatly in many ways, such as their fidelity, response to DNA damage, and conformational dynamics during substrate binding and catalysis. Thus, elucidating the kinetic properties of individual DNA polymerases is an ongoing endeavor.

As the Y-family DNA polymerases are able to bypass various types of DNA lesions *in vitro*, their primary biological role is believed to be catalyzing translesion DNA synthesis (TLS) *in vivo*, a process in which they replicate past damaged DNA bases that would otherwise stall a replication fork.<sup>10</sup> However, when replicating undamaged DNA, the Y-family DNA polymerases display low fidelity and poor processivity and lack the intrinsic proofreading activities that high-fidelity, replicative DNA polymerases utilize to remove misincorporated nucleotides.<sup>11–19</sup> Because of their low nucleotide incorporation fidelities, human Y-family DNA polymerases have been implicated in the incorporation of antiviral nucleoside and nucleotide analogue drugs with unusual chemical structures, potentially contributing to these drugs’ clinical toxicities.<sup>20,21</sup>

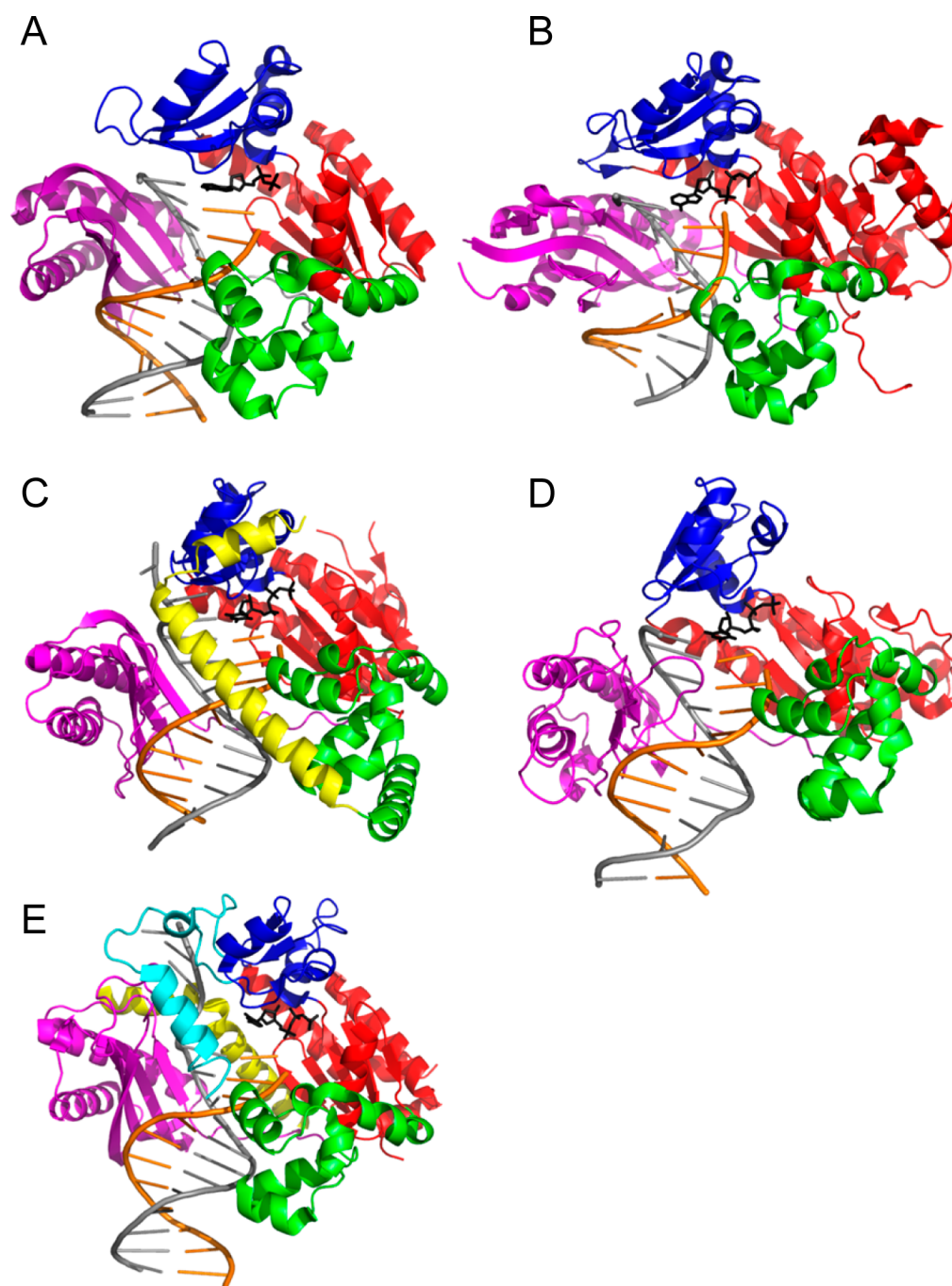
The Y-family DNA polymerases differ structurally from the enzymes in the other families in that the Y-family enzymes contain a unique subdomain termed either the little finger (LF) domain or the polymerase-associated domain (PAD) in addition to the canonical finger, thumb, and palm subdomains (Figure 1).<sup>9,22–24</sup> Interestingly, both the LF subdomain and the linker that connects it to the polymerase core have been implicated in determining the unique lesion bypass properties of a given Y-family DNA polymerase.<sup>23,25</sup> In addition, DNA polymerase  $\kappa$  (pol  $\kappa$ ) also has an N-clasp domain involved in DNA binding (Figure 1C),<sup>26</sup> and Rev1 contains large inserts into the finger and palm domains and an additional N-digit that interacts with incoming nucleotides (Figure 1E).<sup>27</sup> Compared to DNA polymerases in the other families, the Y-family members have more flexible and solvent-accessible active sites that likely allow for the accommodation of various, often bulky, lesions at the expense of the ability to strongly select for correct nucleotides. A thorough review of the structural insights into Y-family DNA polymerases also appears in this issue.<sup>28</sup>

Because of their critical *in vivo* role, the Y-family DNA polymerases have been identified in all three domains of life.<sup>29</sup> Notable family members include *Escherichia coli* DNA polymerases IV (DinB) and V (UmuCD’)<sup>30,31</sup> and human DNA polymerases  $\eta$  (hpol  $\eta$ ),  $\kappa$  (hpol  $\kappa$ ),  $\iota$  (hpol  $\iota$ ), and Rev1 (hRev1).<sup>32</sup> Additionally, DNA polymerase IV (Dpo4) from

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**Figure 1.** Ternary crystal structures of prototype Y-family DNA polymerases in complex with DNA and an incoming nucleotide. Ternary structures of (A) Dpo4 (PDB entry 1JX4), (B) truncated hpol  $\eta$  (PDB entry 3MR2), (C) truncated hpol  $\kappa$  (PDB entry 2OH2), (D) truncated hpol  $\iota$  (PDB entry 1T3N), and (E) truncated hRev1 (PDB entry 3GQC). The finger, palm, thumb, and LF/PAD subdomains are colored blue, red, green, and magenta, respectively. The N-clasp of hpol  $\kappa$  and the N-digit of hRev1 are colored yellow, and an insert into the finger subdomain of Rev1 is colored cyan. The DNA template and primer strands are colored gray and gold respectively, while each incoming dNTP is colored black.

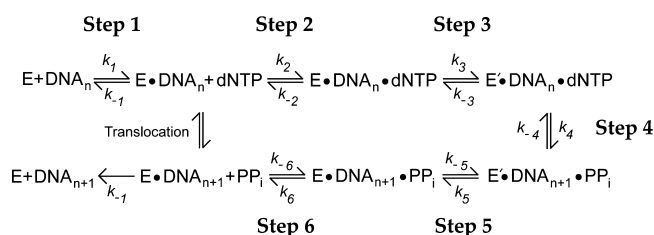
thermophilic archaeon *Sulfolobus solfataricus* has been considered as a model Y-family enzyme because of its high expression levels in *E. coli*, its ease of purification, its high thermostability, bypass abilities similar to those of hpol  $\eta$ , and the fact that it is the only Y-family DNA polymerase encoded by *S. solfataricus*.<sup>33,34</sup> Consequently, Dpo4 has been the most thoroughly investigated Y-family member. Soon after the initial discovery of the Y-family of DNA polymerases, numerous steady-state kinetic studies established that there was great variability among the Y-family members with regard to their preference for

bypassing different types of DNA lesions and their propensity to generate various types of mutations during replication of both damaged and undamaged DNA as reviewed previously.<sup>35,36</sup> Additionally, a wealth of structural information has revealed many details of the diverse strategies that the Y-family DNA polymerases use to accommodate DNA lesions in their active sites and perform catalysis on damaged DNA substrates.<sup>37–39</sup> The primary focus of this review will be on published pre-steady-state kinetic studies in recent years, which

have revealed many new details about individual steps in the varied catalytic pathways of the Y-family DNA polymerases.

## ■ MINIMAL KINETIC PATHWAY FOR NUCLEOTIDE INCORPORATION BY ALL DNA POLYMERASES

Prior to the discovery of the Y-family DNA polymerases, pre-steady-state kinetic studies of numerous model DNA polymerases from the other families and reverse transcriptases<sup>18,19,40–61</sup> had helped to establish a minimal kinetic pathway for nucleotide incorporation consisting of six elementary steps (Figure 2):<sup>4,62</sup> (1) the binding of a DNA



**Figure 2.** Minimal kinetic pathway for nucleotide incorporation catalyzed by DNA polymerases. The elementary steps for nucleotide incorporation common to all DNA polymerases are shown. E and E' represent different conformations of the DNA polymerase, while PP<sub>i</sub> denotes pyrophosphate.

substrate to a DNA polymerase to form the E-DNA binary complex, (2) the binding of a dNTP to form the E-DNA-dNTP loose ground-state ternary complex, (3) the conversion from the ground state to the E'·DNA-dNTP tight activated state with a conformational change in the polymerase, (4) a phosphodiester bond formation step, (5) a reverse protein conformational change step, and (6) the release of pyrophosphate, after which the polymerase can either dissociate from the elongated DNA substrate or remain bound and incorporate additional nucleotides. It is clear that the dissociation of the elongated product from the polymerase limits the multiple turnovers of correct nucleotide incorporation; however, there has been a long-standing debate over whether step 3 or 4 in Figure 2 is rate-limiting during the first turnover.<sup>4,63,64</sup> Various kinetic studies have shown that polymerases may differ substantially in the relative rates of different steps or the inclusion of additional steps in the minimal kinetic mechanism.<sup>4,18,19,40–61,63,64</sup> In comparison, the kinetic studies of incorrect nucleotide incorporation were not as detailed as those of correct dNTP incorporation but did suggest that the chemistry step (step 4 in Figure 2) limits misincorporation in the first turnover.<sup>19,41,65</sup>

## ■ KINETIC MECHANISMS OF REPLICATION OF UNDAMAGED DNA BY THE Y-FAMILY DNA POLYMERASES

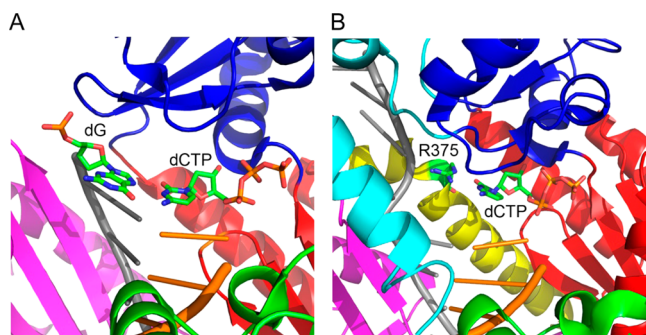
Early pre-steady-state kinetic analyses of yeast pol  $\eta$  (ypol  $\eta$ ), hpol  $\eta$ , and Dpo4<sup>19,51,66,67</sup> suggested that the Y-family DNA polymerases may follow a similar kinetic pathway for correct nucleotide incorporation into undamaged DNA with a rate-limiting, induced-fit conformational change (step 3 in Figure 2), as described previously for all other kinetically characterized DNA polymerases and reverse transcriptases.<sup>18,19,40–61</sup> Interestingly, the conformational change step was also suggested to be rate-limiting for misincorporation for ypol  $\eta$ ,<sup>51</sup> whereas the chemistry step (step 4) was shown to be rate-limiting for incorrect dNTP incorporation in the case of Dpo4.<sup>19</sup> A detailed

study comparing the efficiency of all 16 possibly correct and incorrect nucleotide incorporations revealed that the fidelity of Dpo4 was in the range of  $10^{-3}$  to  $10^{-4}$ , and that the observed fidelity was primarily due to differences in the maximal incorporation rate constants ( $k_p$ ) for correct versus incorrect incorporation rather than differences in nucleotide binding affinities ( $K_D^{\text{dNTP}}$ ).<sup>34</sup> More recent studies have probed details of specific types of mutagenic incorporations by Dpo4.<sup>68,69</sup> In one such study, Dpo4 was shown to accommodate purine-purine mispairs via a Hoogsteen base pairing mechanism in which the incoming dNTP adopts the usual *anti* conformation while the template nucleotide flips into a *syn* orientation.<sup>68</sup> Using a combination of structural insight and pre-steady-state kinetics, it was recently shown that both Dpo4 and hpol  $\kappa$  generate single-base deletions on specific repetitive sequence mutational hot spots through a template slippage mechanism in which the template misaligns with the primer strand prior to nucleotide incorporation.<sup>69,70</sup> However, hpol  $\kappa$  was able to realign the primer and template strands after nucleotide incorporation, resulting in a base substitution rather than a deletion.<sup>70</sup> The mechanism of template-independent, blunt-end nucleotide addition catalyzed by Dpo4 has also been investigated by employing pre-steady-state kinetic methods, and it was demonstrated that dATP addition was preferred because of the favorable intrahelical stacking interactions with both the 5'-base of the opposite strand and the 3'-base of the elongating strand of a DNA blunt end.<sup>71</sup>

Most DNA polymerases have been observed to incorporate nucleotides with a similar fidelity and efficiency for each of the four undamaged template bases. However, pre-steady-state kinetic studies with pol  $\iota$  revealed drastic differences in fidelity and efficiency depending on the identity of the templating base.<sup>72–74</sup> For example, pol  $\iota$  incorporates nucleotides opposite dA with a high fidelity and efficiency and opposite dG and dC with moderate to low fidelity, while incorporation opposite templating dT is highly inefficient and unfaithful, with misincorporation of dGTP significantly favored over correct dATP incorporation.<sup>72–74</sup> Subsequent structural studies were able to provide some insight into the observed differences in kinetics for each template base. Crystal structures show that pol  $\iota$  holds templating purine bases fixed in a *syn* conformation in the dNTP-bound ternary complex, which leads to a Hoogsteen base pairing mechanism for correct nucleotide incorporation, rather than the Watson–Crick base pairing observed for other DNA polymerases (Figure 3A).<sup>75,76</sup> A template dT was observed to maintain an *anti* conformation regardless of the identity of the incoming nucleotide, with an incoming dATP adopting a *syn* conformation with a reduced level of base stacking.<sup>77</sup> In contrast, an incoming dGTP remained in an *anti* conformation opposite the dT template and made an additional stabilizing hydrogen bond to Gln59 of the finger subdomain of pol  $\iota$ , thus explaining the preferential misincorporation of dGTP opposite dT.<sup>77</sup> Recent pre-steady-state kinetic investigation of pol  $\iota$  has further suggested that wobble base pairing or Watson–Crick base pairing may be preferred over Hoogsteen base pairing for some specific dNTP incorporations opposite templating pyrimidines.<sup>78</sup>

Rev1 utilizes perhaps the most unusual nucleotide selection mechanism of any DNA polymerase as it preferentially inserts dCTP opposite all template bases by flipping the templating base out of the active site and instead pairing the incoming dCTP with the side chain of residue R357 in the LF domain (Figure 3B).<sup>27,79–81</sup> Pre-steady-state kinetics have shown that,





**Figure 3.** Noncanonical base pairing in the active sites of hPolI and hRev1. Close-ups of the active sites of (A) truncated hPolI with an incoming dCTP in a Hoogsteen base pair with the templating dG (PDB entry 2ALZ) and (B) truncated hRev1 with an incoming dCTP base pairing with amino acid residue R375 (PDB entry 3GCQ). Protein subdomains and DNA template and primer strands are colored as in Figure 1.

because of the protein template mechanism, Rev1 incorporates dCTP opposite dC, dT, and dA only slightly less efficiently than opposite dG, whereas incorporation of all other dNTPs is significantly less efficient, regardless of the identity of the templating base.<sup>81</sup> Furthermore, the preferential selection of dCTP is believed to be achieved at the nucleotide binding step rather than the incorporation step,<sup>80</sup> and the role of hydrogen bonding between the incoming nucleotide and the side chain of R375 was shown to be more important for catalytic efficiency than base stacking.<sup>81</sup>

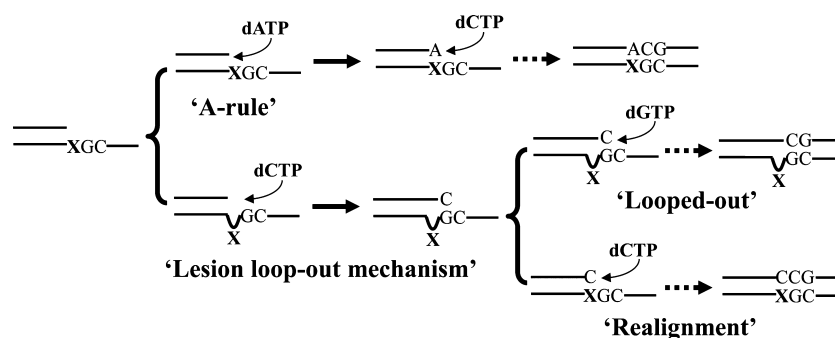
### ■ PRE-STEADY-STATE KINETIC AND SEQUENCING STUDIES OF THE MECHANISMS AND MUTAGENIC PROFILES OF DNA LESION BYPASS

Steady-state kinetics provided initial qualitative insight into TLS by the Y-family DNA polymerases.<sup>35,36</sup> More recent pre-steady-state kinetic studies have provided the necessary detailed insight into the kinetic mechanisms of lesion bypass and have illustrated that some DNA lesions are bypassed with kinetic parameters similar to those of undamaged DNA for a given Y-family DNA polymerase, while other lesions may have drastic consequences for nucleotide insertion kinetics.<sup>82–94</sup> With the exception of two studies with ypol  $\eta$ ,<sup>82,83</sup> the majority of the early pre-steady-state kinetic studies of TLS were performed by using Dpo4 as a model enzyme.<sup>84–90</sup> Notably, although *S. solfataricus* grows optimally at temperatures of  $\geq 80$  °C, temperature-dependent studies showed that the nucleotide incorporation fidelity, induced-fit mechanism, and secondary

structural features of Dpo4 remained unchanged over a wide range of temperatures,<sup>95,96</sup> validating the appropriateness of comparison between Dpo4 and other Y-family DNA polymerases at 37 °C. As initially observed for the bypass of a *cis-syn* thymine-thymine (TT) dimer and a 7,8-dihydro-8-oxoguanine (8-oxoG) by ypol  $\eta$ ,<sup>82,83</sup> Dpo4 was shown to bypass an 8-oxoG lesion with  $K_D^{dNTP}$  and  $k_p$  values similar to those for the analogous undamaged DNA bases, indicating that the kinetic mechanism was not significantly perturbed by this lesion.<sup>87,88</sup> In fact, these studies demonstrated that Dpo4 bound to both correct dCTP and DNA more tightly with 8-oxoG as the templating base as compared to unmodified dG, whereas the efficiency of incorporation opposite the related  $O^6$ -methylguanine lesion is slightly reduced.<sup>89</sup> In contrast, in running start assays with Dpo4 where a stretch of a template is replicated in the presence of all four dNTPs starting several positions upstream from a DNA lesion, a significant accumulation of intermediate products corresponding to incorporations opposite and adjacent to the lesion has been observed for a number of DNA lesions.<sup>85,90,97–102</sup> The observation of these “polymerase pause sites” indicates that, while these DNA lesions can be traversed by Dpo4, the significant slowing of the progress of DNA synthesis is a general mechanism for the bypass of more disruptive DNA lesions.

Pre-steady-state kinetic methods have been used to provide detailed insight into how DNA lesions alter the kinetics of nucleotide incorporation by Dpo4 at these pause sites.<sup>85,90,97,98</sup> For example, a comprehensive pre-steady-state kinetic investigation showed that the kinetic efficiency ( $k_p/K_D^{dNTP}$ ) of Dpo4 was reduced by up to 3 orders of magnitude at the two strong pause sites corresponding to nucleotide incorporation opposite and extending from a noncoding abasic site due to a strong reduction in both the  $k_p$  and nucleotide binding affinity ( $1/K_D^{dNTP}$ ) compared to those of correct incorporation into undamaged DNA.<sup>90</sup> The authors also demonstrated that Dpo4 utilized two competing mechanisms for abasic site bypass: an “A-rule” mechanism in which dATP was preferentially incorporated opposite the lesion or a “lesion loop-out” mechanism in which the template base 5' to the lesion dictates nucleotide incorporation preference (Figure 4).<sup>90</sup> Subsequently, two analogous incorporation modes were also proposed for the bypass of a benzo[*a*]pyrene-derived  $N^2$ -dG adduct by Dpo4 in certain sequence contexts.<sup>86</sup>

Further branching of the lesion bypass pathway occurs during nucleotide incorporations following the initial lesion loop-out in which the damage base can remain looped-out, leading to a frameshift mutation, or the primer can realign with

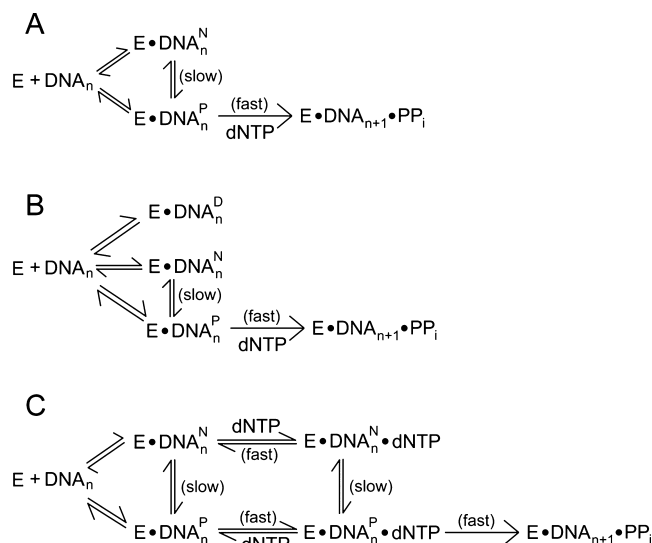


**Figure 4.** Competing pathways for abasic lesion bypass catalyzed by Dpo4. An example of a branched pathway for lesion bypass is shown where the identity of the incorporated nucleotide determines which bypass mechanism is utilized. X denotes an abasic site.

the template, allowing the previously incorporated base to be positioned opposite the lesion (Figure 4). In the case of Dpo4 bypassing an abasic site, the pathway in which the lesion remained looped-out was shown to be dominant over realignment.<sup>90</sup>

To verify the predictions of kinetic studies regarding the types and frequencies of the various products generated during TLS, a short oligonucleotide sequencing assay (SOSA) method was developed to directly determine the sequences of individual products generated during TLS through abasic sites by Dpo4.<sup>103</sup> This novel methodology was used to confirm the relative frequencies of insertion of dATP versus insertion of dCTP via the lesion loop-out mechanism predicted from the kinetic efficiencies of each pathway and to provide information about more rare mutation events both opposite and downstream from the lesion.<sup>103</sup> The development of “next-generation sequencing” allows for a high-throughput alternative to the original SOSA method and has been used to assess the mutagenic profiles of several carboxymethylated DNA lesions in *E. coli*.<sup>104</sup> In a recent publication, novel software (Next-Generation Position Base Counter, available for download at <https://chemistry.osu.edu/~suo.3/index.html>) was developed to allow for efficient analysis of millions of DNA sequences yielded from the next-generation sequencing-based high-throughput version of the SOSA technique (HT-SOSA).<sup>105</sup> This new methodology provides a powerful and cost-effective tool for investigating the mutagenic potential of various types of DNA damage as demonstrated by its use in the analysis of the products of *cis-syn* TT dimer bypass catalyzed by human Y-family DNA polymerases.<sup>105</sup>

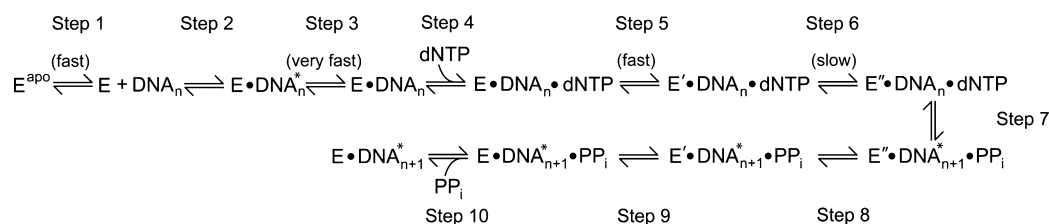
Performing single-turnover kinetic experiments in the presence of an unlabeled DNA trap, which removes all polymerase molecules that dissociated from the <sup>32</sup>P-labeled, lesion-containing DNA substrate prior to mixing with correct dNTP, can provide important insight into nucleotide incorporation mechanisms during lesion bypass and subsequent extension. In this trap DNA assay with Dpo4, biphasic kinetics were observed for nucleotide incorporation opposite both an abasic lesion and the next template base.<sup>90</sup> The observation of a small reaction amplitude in the fast phase and a large reaction amplitude in the slow phase was interpreted to represent a mechanism in which Dpo4 could form either a productive  $E \cdot DNA_n^P$  binary complex or a distinct nonproductive  $E \cdot DNA_n^N$  complex that could be slowly converted to the productive complex or dissociate (Figure 5A). Similar studies of the bypass of various *N*<sup>2</sup>-alkylguanine adducts,<sup>84</sup> a 3-nitrobenzanthrone adduct,<sup>97</sup> or a double-base cisplatin–d(GpG) adduct<sup>85</sup> by Dpo4 suggested that this two-species binary complex pathway may be a general mechanism for TLS, though significant differences were observed in the relative reaction amplitudes of the two phases depending on the size and position of the adduct or of the first and second cross-linked base in the cisplatin–d(GpG) adduct. Notably, this type of biphasic kinetics is not observed with undamaged DNA or 8-oxoG,<sup>88</sup> indicating that this mechanism is unique to the bypass of more disruptive DNA lesions. Evidence of a third “dead-end” binary complex that could not be converted to the active complex was found for the bypass of a bulky *N*-(deoxyguanosin-8-yl)-1-aminopyrene (dG<sup>AP</sup>) adduct by Dpo4 due to the observation that a large percentage of dG<sup>AP</sup>-containing DNA substrate was never elongated in the presence of a DNA trap (Figure 5B).<sup>98</sup> However, an increase in the rate of dissociation of the nonproductive complex is another possible interpretation of



**Figure 5.** Kinetic schemes derived from biphasic kinetic analysis. (A) Kinetic scheme for lesion bypass, including productive and nonproductive binary complexes. Expanded schemes also include the possibility of a dead-end binary complex (B) or productive and nonproductive ternary complexes (C).

this result. X-ray crystallographic studies<sup>101,106,107</sup> have supported these conclusions by demonstrating that the bulky hydrocarbon adducts of several lesions, including dG<sup>AP</sup>, adopt multiple conformations relative to the active site of Dpo4 corresponding to the productive, nonproductive, and dead-end complexes inferred from the aforementioned kinetic studies. Structural evidence also suggests that *ypol*  $\eta$  can potentially adopt productive and nonproductive ternary complexes during the bypass of a cisplatin adduct.<sup>108</sup> Because of rapid nucleotide binding, the biphasic kinetic assays cannot distinguish between binary and ternary complexes in the productive or nonproductive states, and thus, an expanded kinetic scheme was proposed to allow for the possibility of productive and nonproductive states in both binary and ternary complexes (Figure 5C).<sup>85</sup> Interestingly, pre-steady-state kinetic studies suggest that *hpol*  $\eta$  exhibits the biphasic kinetic behavior during incorporation opposite both dG<sup>AP</sup> and an unmodified dG, though the full implications of this observation in the case of the undamaged DNA are unclear.<sup>91</sup>

With a foundation established for the kinetics of TLS by Dpo4, more recent work has aimed to investigate the mechanistic differences in the ability (or inability) of each of the four human Y-family DNA polymerases to bypass various lesions. For example, steady-state and pre-steady-state kinetic studies indicated that both *hpol*  $\eta$  and *hpol*  $\kappa$  can bypass an M<sub>1</sub>dG [3-(2'-deoxy- $\beta$ -D-erythro-pentofuranosyl)pyrimido[1,2-*a*]purin-10(3*H*)-one] adduct, while *hpol*  $\iota$  and *hRev1* cannot.<sup>92</sup> In the case of an abasic site, while all human Y-family enzymes were able to incorporate dNTPs opposite the lesion,<sup>93,94</sup> both the kinetic efficiency of lesion bypass and the mutagenic profiles derived from SOSA assays indicate that *hpol*  $\eta$  is the most suitable polymerase for bypassing abasic lesions *in vivo*.<sup>94</sup> An exhaustive study of the pre-steady-state kinetics of dG<sup>AP</sup> bypass polymerases illustrated many differences in the kinetic mechanisms of the four human Y-family enzymes.<sup>91</sup> This study showed that both fidelity and efficiency were reduced for all four human Y-family enzymes during both incorporation opposite the lesion and extending from the dG<sup>AP</sup>:dC base



**Figure 6.** Expanded kinetic mechanism of correct nucleotide incorporation catalyzed by Dpo4.  $E \cdot DNA_n^*$  ( $E \cdot DNA_{n+1}^*$ ) and  $E \cdot DNA_n$  represent pre- and post-translocation binary complexes, respectively.  $E^{apo}$ ,  $E$ ,  $E'$ , and  $E''$  represent conformations of Dpo4 in the apo state, the DNA-bound binary complex, after the precatalytic domain motion, and after the active site rearrangement, respectively.  $PP_i$  denotes pyrophosphate. The relative speeds of the forward rates for several of the conformational changes steps are indicated.

pair. It was also shown that the decrease in efficiency was primarily due to the drastically reduced  $k_p$ , especially for hpol  $\kappa$  and hpol  $\iota$ , which was measured to be up to  $\sim 1000$ -fold slower than for undamaged DNA. In a follow-up study using the previously developed SOSA method, the bypass of  $dG^{AP}$  by the human Y-family enzymes was shown to be very mutagenic, with a high frequency of deletion mutations.<sup>109</sup> Interestingly, despite the fact that the kinetic studies revealed hpol  $\eta$  to be the most efficient in bypassing and extending from the  $dG^{AP}$  lesion,<sup>91</sup> the mutagenic profiles suggested that *in vivo* bypass of the lesion might primarily involve the incorporation of dCTP opposite the adduct by hRev1 followed by an extension of the lesion bypass product by hpol  $\kappa$ .<sup>109</sup> Notably, hRev1 may play a general role in bypassing many guanine adducts because of its strong preference for incorporating dCTP regardless of the identity of the templating base, and it has been shown to have only moderately reduced efficiency when replicating bulky  $N^2$ - and  $O^6$ -alkylguanine DNA adducts.<sup>110</sup>

## CONFORMATIONAL CHANGES REVEALED BY CRYSTAL STRUCTURES

Upon binding to an incoming nucleotide, many DNA polymerases have been shown to undergo a finger subdomain closing conformational change that helps bring functionally important enzyme residues into contact with the nucleotide.<sup>111</sup> This transition was first observed by comparing X-ray crystal structures of binary complexes ( $E \cdot DNA$ ) and ternary complexes ( $E \cdot DNA \cdot dNTP$ )<sup>5,6,112</sup> and was initially believed to represent the rate-limiting conformational change step (step 3 in Figure 2) inferred from kinetic studies. However, solution-based pre-steady-state fluorescence studies with pol  $\beta$  demonstrated that this conformational change was fast and occurred prior to the rate-limiting step.<sup>59,60</sup> Subsequently, stopped-flow fluorescence studies also suggested that the finger subdomain closure was too rapid to be the rate-limiting step for Taq DNA polymerase,<sup>61,113,114</sup> T7 DNA polymerase,<sup>115</sup> and HIV reverse transcriptase.<sup>116</sup>

Notably, a large finger domain motion upon nucleotide binding is not observed in crystal structures of the Y-family DNA polymerases.<sup>9,117</sup> However, a large conformational change does occur from the apo state to DNA-bound binary state characterized by a rotation of the LF domain relative to the polymerase core for some Y-family polymerases, including Dpo4, pol  $\kappa$ , and to a lesser extent pol  $\eta$ .<sup>24,26,108,118–120</sup> Also, in contrast to replicative DNA polymerases, binary structures of Dpo4 show that the terminal base pair of the DNA substrate occupies the binding pocket for an incoming nucleotide, implying that the DNA must translocate by 1 bp relative to Dpo4 to allow for dNTP binding.<sup>9,117</sup> Further investigation of the potential conformational changes of the Y-family DNA

polymerases in the solution state has been an area of active research in recent years as discussed in the following section.

## SOLUTION-STATE CONFORMATIONAL DYNAMICS DURING dNTP INCORPORATION

Despite the lack of crystallographic evidence of a large finger subdomain motion upon nucleotide binding, several stopped-flow fluorescence studies have provided information about various conformational change steps during nucleotide incorporation by the Y-family DNA polymerases,<sup>121–124</sup> leading to the development of an expanded minimal kinetic pathway (Figure 6).<sup>122,124</sup> One such study using DNA labeled with the fluorescent reporter 2-aminopurine (2-AP) suggests the existence of several rapid noncovalent steps between the initial binding of dNTP and the covalent incorporation step in the catalytic mechanism of Dbh, the Dpo4 homologue from *Sulfolobus acidocaldarius*.<sup>121</sup> While the exact nature of these steps was unclear, they were interpreted to result from rearrangements in the polymerase active site necessary to properly align catalytic residues, metal ions, and substrate functional groups for catalysis. Another study revealed several conformational change steps during nucleotide incorporation by Dpo4 by monitoring fluorescence from Trp introduced at residues distant from the active site.<sup>123</sup> This study suggested that Dpo4 does indeed undergo a precatalytic protein conformational change too rapid to be rate-limiting, along with a slower reverse conformational change after the chemistry step. To provide more detailed information about the nature of these putative conformational changes, a thorough stopped-flow Förster resonance energy transfer (FRET)-based study was conducted to monitor the motions of each subdomain of Dpo4 during correct nucleotide incorporation.<sup>122</sup> The results from the FRET investigation showed that the pre- and postcatalytic conformational changes involve motions of all four subdomains of Dpo4 relative to a fluorophore on the DNA substrate. Because the precatalytic conformational change associated with these domain motions was rapid (step 5 in Figure 6), a rearrangement of the active site residues was instead proposed to limit the rate of correct nucleotide incorporation (step 6 in Figure 6).<sup>122</sup> Additionally, by monitoring multiple sites in each protein subdomain, this study provided information about the rotational and translational aspects of each subdomain's motion. This allowed for the subsequent identification of alterations in the structural nature of the conformational changes in the finger, thumb, and palm subdomains during nucleotide incorporation opposite and extending from an 8-oxoG lesion,<sup>125</sup> whereas no such differences in conformational changes during incorporation into 8-oxoG-containing or undamaged DNA could be detected by monitoring only Trp fluorescence.<sup>123</sup> However, more recent



Trp fluorescence studies have indicated that bulky N<sup>2</sup>-alkylguanine adducts can impair the ability of Dpo4 to undergo precatalytic conformational changes, while smaller adducts at the same position have only minor effects on the rate of conformational change.<sup>126</sup> These stopped-flow studies have provided the first evidence to suggest the Y-family DNA polymerases may modify their conformational change steps to accommodate various types of DNA damage, and investigations are underway to determine the effects of other DNA lesions on the conformational changes of Dpo4.

A recent study used an interdomain FRET system to monitor the motion of the LF subdomain relative to the polymerase core during DNA binding,<sup>124</sup> which had previously been observed in crystal structures.<sup>118</sup> The results from this study suggest that the conformational change of the LF subdomain occurs as a relatively fast step distinct from the initial binding or dissociation of the Dpo4·DNA complex (step 1 in Figure 6). In addition to the conformational change in the protein, the results of the 2-AP fluorescence study proposed an expansion of the DNA binding step by including an equilibrium between pre- and post-translocation conformations of the DNA substrate in the Dpo4·DNA binary complex<sup>121</sup> inferred from binary crystal structures in which the terminal base pair occludes the dNTP binding site (step 3 in Figure 6).<sup>9,117</sup> Similarly, using the FRET methodology, an observed very rapid FRET decrease phase was interpreted to represent this shift in equilibrium toward the post-translocation state induced by nucleotide binding.<sup>122</sup> Furthermore, when extending from an 8-oxoG:C base pair, this translocation step was significantly slowed compared to that for undamaged DNA,<sup>125</sup> perhaps because of an increased level of hydrogen bonding between Arg 331 and Arg 332 residues in the LF domain of Dpo4 and the modified base.<sup>117</sup>

Beyond stopped-flow fluorescence methods, several additional biophysical techniques offer the potential for gaining further understanding of the conformational dynamics of the Y-family DNA polymerases. For example, molecular dynamics simulations with Dpo4 have revealed subtle but potentially important conformational changes in the LF and finger subdomains upon the release of catalytic metal ions prior to catalysis that may be involved in repositioning the DNA substrate to allow for different types of DNA lesions in the active site.<sup>127</sup> More recently, hydrogen–deuterium exchange in tandem with mass spectrometry has been used to demonstrate changes in flexibility in certain regions of the finger and thumb subdomains of Dpo4 that are involved in correct nucleotide selection.<sup>128</sup> In another recent publication, the combination of femtosecond fluorescence spectroscopy and molecular dynamics simulation was used to probe the solvent dynamics in apo, binary, and ternary Dpo4 complexes.<sup>129</sup> The results from this study suggested that a hydrated binding interface facilitates the sliding of Dpo4 on the DNA substrate to allow for rapid translocation and that the dynamic solvent accessibility of the active site contributes to the low fidelity of Dpo4.<sup>129</sup> Additionally, the recent chemical shift backbone assignment of the polymerase core<sup>130</sup> and LF subdomain<sup>131</sup> of Dpo4 will facilitate the investigation of conformational dynamics at atomic-level resolution via protein NMR spectroscopy. Single-molecule FRET investigations have also begun to provide new insights into the kinetic mechanisms of several canonical DNA polymerases<sup>132–137</sup> and may prove to be useful in studying the Y-family DNA polymerases, as well.

## CONCLUDING REMARKS

Y-Family DNA polymerases are fascinating enzymes that exhibit many unique catalytic properties because of their ability to accommodate a wide range of modified DNA substrates in their active sites. Pre-steady-state kinetic methods have begun to reveal some of the distinctive mechanistic details for this interesting class of enzymes and have shed light on how they alter their kinetic properties and conformational dynamics during replication of damaged and undamaged DNA. However, many questions remain unanswered, particularly with regard to how the kinetic and structural properties of the Y-family DNA polymerases influence the regulation of switching between the replicative and Y-family DNA polymerases before and after the bypass of DNA lesions to ensure that undamaged DNA is copied with high fidelity. Additionally, the Y-family DNA polymerases may play critical roles in several cellular processes beyond TLS. To address these lingering concerns, the Y-family DNA polymerases are sure to be the subject of many future investigations, especially as new biophysical and biochemical techniques evolve to allow for deeper probing of enzyme mechanisms and conformational dynamics.

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## ABBREVIATIONS

2-AP, 2-aminopurine; 8-oxoG, 7,8-dihydro-8-oxoguanine; dG<sup>AP</sup>, N-(deoxyguanosin-8-yl)-1-aminopyrene; DinB, *E. coli* DNA polymerase IV; Dpo4, *S. solfataricus* DNA polymerase IV; FRET, Förster resonance energy transfer; hPol $\eta$ , human DNA polymerase  $\eta$ ; hPol $\iota$ , human DNA polymerase  $\iota$ ; hPol $\kappa$ , human DNA polymerase  $\kappa$ ; hRev1, human Rev1; LF, little finger; PAD, polymerase-associated domain; PDB, Protein Data Bank; SOSA, short oligonucleotide sequencing assay; TT, *cis-syn* thymine-thymine dimer; TLS, translesion DNA synthesis; UmuCD', *E. coli* DNA polymerase V; yPol $\eta$ , yeast DNA polymerase  $\eta$ .

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