Conformational Changes during Nucleotide Selection by 
Sulfolobus solfataricus DNA Polymerase Dpo4

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The mechanism of nucleotide selection by Y-family DNA polymerases has been the subject of intense study, but significant structural contacts and/or conformational changes that relate to polymerase fidelity have been difficult to identify. Here we report on the conformational dynamics of a model Y-family polymerase Dpo4 from Sulfolobus solfataricus. Hydrogen-deuterium exchange in tandem with mass spectrometry was used to monitor changes in Dpo4 structure as a function of time and the presence or absence of specific substrates and ligands. Analysis of the data revealed previously unrecognized structural changes that accompany steps in the catalytic cycle leading up to phosphoryl transfer. For example, the solvent accessibility of the αB-loop-αC region in the finger domain decreased in the presence of all four dNTP insertion events, but the rate of deuterium exchange, an indicator of conformational flexibility, only decreased during an accurate insertion event. Of particular note is a change in the region surrounding the H-helix of the thumb domain. Upon binding DNA and Mg²⁺, the H-helix showed a decrease in solvent accessibility and flexibility that was relaxed only upon addition of dCTP, which forms a Watson-Crick base pair with template dG and not during mispairing events. The current study expands upon a previous report from our group that used a fluorescent probe located near the thumb domain to measure the kinetic properties of Dpo4 conformational changes. We now present a model for nucleotide selection by Dpo4 that arises from a synthesis of both structural and kinetic data.

The mechanisms utilized by DNA polymerases to catalyze replication and/or repair of genomic material provide a fascinating example of how an enzyme can select a single substrate from multiple candidates, all with similar structural and chemical properties. DNA polymerases inside the cells of every living organism select from a pool of (four) dNTP substrates to catalyze phosphoryl transfer and then extend from a nascent primer strand opposite a DNA template. It has been emphasized that the endpoint of each nucleotide selection event cannot be determined solely by the thermodynamics of a given base pair (1, 2). Instead, the molecular features intrinsic to DNA polymerases are generally thought to guide the free-energy landscape of phosphoryl transfer toward the selection of “Watson-Crick” pairs (3, 4), at least among the four canonical bases. It is now apparent that individual DNA polymerases use variations upon a general mechanism to determine nucleotide selectivity (4–8). However, the mechanistic differences that define each polymerase class are only now being elucidated in any molecular detail.

The Y-family DNA polymerases (pols)³ represent one class of polymerases with distinctive structural and functional features (8, 9). Like most other DNA polymerases, the Y-family pols have three domains: the finger for dNTP selection, the palm for catalysis, and the thumb for double strand DNA contact/orientation, which together form a structure that has been likened to a right hand. The Y-family polymerases also possess a unique domain that has been called the little finger or palm-associated domain. Most of the additional domains beyond these four core domains are involved in a complex web of protein-protein interactions and cellular localization events, although there are clearly exceptions to this rule (e.g. the N-clasp of pol k and the N-digit of REV1) (10, 11). The Sulfolobus solfataricus DNA polymerase Dpo4 has served as the prototypical Y-family polymerase, because it is especially amenable to structural analysis and it shares many features and properties of other Y-family members. Numerous Dpo4 crystal structures have been reported in the literature (12–18). However, structural alignments of binary and ternary structures have failed to reveal the “open” to “closed” transition observed for some other pols (19). It is clear that, upon binding DNA, the little finger of Dpo4 probably undergoes a dramatic translation/rotation through space (20, 21), but few other conformational changes have been observed. The lack of obvious conformational rearrangements has led to the proposal that the Y-family DNA polymerases possess a “pre-formed” active site (22). However, there is substantial kinetic evidence in support of the view that a non-covalent step or steps occur in the Dpo4 reaction cycle prior to nucleophilic attack upon (what is assumed to be) a deprotonated 3′-hydroxyl group at the primer terminus, at least during

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The abbreviations used are: pol, (DNA) polymerase; DTT, dithiothreitol; ESI, electrospray ionization; HDX, hydrogen-deuterium exchange; LC, liquid chromatography; HPLC, high performance LC; UPLC, ultraperformance LC; MS/MS, tandem mass spectrometry; MS, mass spectrometry; 8-oxoG, 7,8-dihydro-8-oxo-2′-deoxygenosine; pol T7, bacteriophage pol T7 (exonuclease-deficient); UDG, uracil DNA glycosylase.
formation of Watson-Crick geometry (23, 24). Previous work from our group used tryptophan fluorescence to monitor changes during dNTP insertion and following phosphoryl transfer (25). In the present study we sought to identify structural changes that occur during the Dpo4 reaction cycle by using hydrogen-deuterium exchange in tandem with mass spectrometry (HDX-MS). The method of HDX-MS has been used successfully to probe conformational dynamics of proteins and enzymes (26–29). The temporal and structural resolution provided by HDX-MS can serve as an effective bridge between kinetic analysis, which provides evidence of changes but cannot identify locations, and the inherently static nature of crystal structures. Our results provide new insight into Y-family polymerase catalysis, which may lead to a better understanding of how these enzymes select nucleotide substrates and, ultimately, how they contribute to mutagenesis.

**EXPERIMENTAL PROCEDURES**

_Materials—_Wild-type Dpo4 was expressed in *Escherichia coli* and purified to electrophoretic homogeneity as described previously (17). Following storage in Tris–HCl (pH 7.4) containing 1 mM DTT, 1 mM EDTA, and 50% glycerol (v/v), the enzyme was dialyzed against 500 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 50% glycerol (v/v) overnight. The concentration of the enzyme following dialysis was estimated by measuring the absorbance at 280 nm ($\epsilon_{280} = 24,100 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The dialyzed protein was filtered through a 0.2-$\mu$m syringe tip filter and aliquoted into 100-$\mu$l samples (160 $\mu$m final concentration), flash-frozen in dry ice/ethanol, and stored at −80 °C. Samples were thawed and used for HDX experiments on the same day. Pepsin was purchased from Worthington Biochemicals (Lakewood, NJ) and dissolved in 10 mM ammonium acetate (pH 5.0) just prior to performing the hydrogen-deuterium exchange reactions. All unlabeled dNTPs were obtained from Amersham Biosciences. All oligonucleotides used in this work were synthesized by Midland Certified Reagent Co. (Midland, TX) and purified by the manufacturer using high-performance liquid chromatography, with analysis by matrix-assisted laser desorption time-of-flight MS. The 13-base primer sequence used in this work was corrected for deuterium loss during digestion and UPLC separation (i.e., “back-exchange”) by using a 6-h time point ($m_{m/z}$) as a reference for each experimental condition, which should take into account experimental variability from one run to the next. LC-MS/MS analysis was performed as described above with the following distinctions. For HDX experiments the autosampler was cooled to 5 °C and the injector bypassed so that the UPLC column could be packed in ice. Solvents were kept on ice during the analysis. The following gradient program was used with a flow rate of 50 $\mu$l min$^{-1}$: 0–3 min, linear gradient from 100% buffer A (see above) to 97%A/3%B (v/v); 3–4.5 min, linear gradient to 80%A/20%B (v/v); 4–5.5 min, linear gradient to 100%B; 5–5.5 min, hold at 100%B; 5.5–6.5 min, linear gradient to 100%A; 6.5–9.5 min, hold at 100%A. The column was placed in ice for the duration of the experiment. ESI conditions were as described above.

Quantification of Deuterium Incorporation—Data sets were analyzed in Matlab (Mathworks) using a modified version of the DoGEX software developed by our group (30). Peaks defining the isotopic envelope in a given $m/z$ and time range were detected. A weighted average was used to deconvolute the peptide envelope (supplemental Fig. S1). The deconvolution was calculated by summing all the isotopic intensities in the envelope and multiplying them by their respective $m/z$ values. The $m/z$ intensity sum was divided by the sum of all the intensities.
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Each deconvoluted m/z value corresponded to a different degree of deuterium incorporation corresponding to a particular time in the reaction for a given peptide. The deuterium content of each peptide was adjusted for gain or loss during the experiment using Equation 1,

\[ D = \frac{(m_t - m_{0\%})/(m_{100\%} - m_t)}{N} \times N \]  
(Eq. 1)

where \( D \) is the deuterium content in a given peptide and \( m_0 \), \( m_{0\%} \), and \( m_{100\%} \) are the weighted average m/z values calculated for a peptide at a given time \( (m_t) \), for the exchange-in experiment \( (m_{0\%}) \), and for a maximally deuterated sample \( (m_{100\%}) \), respectively. The total number of amide protons \( N \) depends upon the peptide sequence \( (N = \text{length of peptide} - \text{(N terminus)} - \text{(any prolines)}) \). The number of deuterons incorporated was plotted as a function of time, and individual data sets were fit using GraphPad Prism (GraphPad Software, La Jolla, CA) to the sum of first-order rate terms (Equation 2).

\[ D = N - \sum_{j=1}^{N} \exp(-k_j t) \]  
(Eq. 2)

The structural representations were prepared using Pymol (44).

RESULTS

Peptide Identification and Rapid Exchange Maps—Dpo4 was initially incubated with pepsin and \( \text{H}_2\text{O} \) to identify the peptides generated during the digestion step. A long chromatographic step was coupled with data-dependent fragmentation of the resulting peptides. The resulting MS/MS spectra were submitted to the Mascot server (Matrix Science) to identify Dpo4 peptides based on their fragmentation patterns. The resulting map gave 96% coverage of the 352 amino acids that comprise the Dpo4 polypeptide (supplemental Table S1). However, during the HDX analysis a shorter chromatographic gradient was used upon hydrogen bond participation. Changes in the measured rates of HDX for a peptide may represent changes in either the stability/identity of hydrogen bonding patterns, which may include spatial rearrangements. Either of these events is indicative of changes in the structure of the protein. A qualitative picture of the number of rapidly exchanging hydrogens \( (k > 4 \text{ min}^{-1}) \) can be obtained by measuring the amount of HDX at a relatively short time scale \( (\text{e.g.} 15 \text{ s}) \). It seems intuitive that the exposure to solvent would be reflected in the rate of rapid HDX, and if this were true then identifying regions that have rapidly exchanging hydrogens would be indicative of solvent exposure. However, there is evidence in the literature that HDX is largely insensitive to the degree of solvent exposure for backbone hydrogens (31). A "rapid exchange map" is therefore a very rough estimate of solvent accessibility at best. Moreover, although a single time point is inextricably linked to the kinetics, it is not necessarily predictive of either the rates obtained when fitting a time course to multiple exponential equations or the final amplitudes of HDX observed in time course experiments. In the case of Dpo4, the rapid exchange map is largely confirmatory in nature because of the large amount of structural information already present in the literature. These so-called "rapid exchange" maps therefore provide an initial overview of deuterium exchange occurring under a given set of experimental conditions \( (\text{i.e. with or without DNA and/or dNTPs}) \). The rapid exchange map for Dpo4 in isolation indicated that, unsurprisingly, the palm domain exhibited the least amount of exchange in the first 15 s of the reaction (Fig. 1B, and supplemental Table S1). Portions of the finger and thumb domains exhibited the highest amounts of deuterium incorporation for the apoenzyme (Fig. 1B).

The addition of primer/template DNA and \( \text{MgCl}_2 \) reduced the fraction of rapid exchange by \( >5\% \) for 12 peptides (Fig. 2 and Table 1). One peptide located in the linker region between the thumb and little finger domains (residues 229–239) exhibited a 12% increase in the amount of rapid exchange observed (Fig. 2 and Table 1). Overall, the protected regions aligned well with what is observed in the ternary crystal structures of Dpo4 bound to primer/template DNA (Fig. 2). For example, helices H, J, and K (represented by peptides that contain residues 180–202, 210–218, and 215–229, respectively) all exhibited a decrease in rapid exchange upon addition of primer/template DNA and \( \text{MgCl}_2 \). The loop region located between helices B and C in the finger domain (residues 48–63) was protected from deuterium exchange upon addition of DNA substrate, as are residues 272–293 in the little finger domain (Fig. 2 and Table 1).

The addition of dCTP resulted in changes of \( >5\% \) of the maximal deuterium incorporation for eight peptides, but these were not the same set of peptides noted following addition of primer/template DNA and \( \text{MgCl}_2 \) (Fig. 2 and Table 1). It should be mentioned again that 2’,3’-dideoxy-terminated primers were used in all experiments that contain dNTP in the reaction mixture. The lack of a 3’-hydroxyl moiety prohibited phosphodiester bond formation from occurring in the HDX experiment. Six of the peptides that changed upon addition of dCTP showed a decrease in the rapid phase of exchange, but two peptides in the thumb domain (residues 180–202 and 215–229) showed an increase in rapid exchange upon addition of dCTP. With the exception of one peptide in the palm domain (residues 151–170 comprising the F-helix), the peptides that exhibited changes in the rapid HDX phase upon addition of dCTP are found in the finger and thumb domain.

Changes in HDX Specific to Nucleotide Selectivity—Separate HDX time courses were performed with either Dpo4 alone, with DNA/\( \text{Mg}^{2+} \), or with DNA/\( \text{Mg}^{2+} \)/dNTP (supplemental Fig. S2 and Tables S3–S5). In the sequence context used for the HDX experiments, dCTP would represent a normal Watson-Crick pairing mode for insertion, and binding of dGTP would most likely result in formation of a “Type II” mode of binding (14) where the incoming nucleotide skips the template base at
position 0 and pairs with the base at position +1 (5’ to position 0). Inclusion of either dATP or dTTP in the HDX experiment is expected to yield such mispairing events.

The majority of the protein did not exhibit changes in HDX during dCTP binding (supplemental Fig. S2 and Tables S3–S5), but some notable changes were observed in the Dpo4 HDX
Dpo4 Conformational Dynamics Using H-D Exchange

The models describing a simple open and closed polymerase structure determining fidelity have been supplanted by more refined descriptions of the molecular events associated with fidelity (32). In the past, most reaction schemes describing polymerase catalysis include a non-covalent step prior to phosphoryl transfer with no distinction made for accurate or error-prone insertion events. It has recently been shown that for bacteriophage T7 DNA polymerase (pol T7) there are different conformational states for correct and incorrect substrates (4). Kinetic and structural analysis by our own group has illus-

kinetics following addition of individual dNTPs. First, the loop region between helix B and C in the finger domain (residues 48–63) exhibited changes in both the amount and rate of deuterium incorporated when an incoming dNTP was present (Fig. 3, A and B). All four dNTPs caused reductions in the amount of deuterium incorporated, which could indicate decreased solvent accessibility and/or a decrease in the number of hydrogen bonding interactions. A decrease in the HDX rate was only observed upon addition of either dCTP or (to a lesser extent) dGTP (Fig. 5A and Table 2). The decrease in the rate of exchange was lost upon addition of other dNTPs. Inspection of the HDX kinetic profiles revealed that the K-helix exhibits more stable and/or numerous hydrogen bonding interactions upon binding to DNA/Mg2+ and either dATP or dTTP (Fig. 5A and Table 2). The decrease in the rate of exchange was lost upon addition of either dCTP or (to a lesser extent) dGTP (Fig. 5A and Table 2). The K-helix, which is in close proximity to the H-helix in the Dpo4 structure, also exhibited an increase in HDX when dCTP was present, relative to was observed with only DNA and Mg2+ (Fig. 5B and Table 2). These two helices in the thumb domain appeared to be sensitive to the identity of the incoming nucleotide, and their importance regarding polymerase fidelity and kinetics is discussed below.

DISCUSSION

The loop region containing residues 48–63 is in contact with what has been termed the “roof” of the Dpo4 active site (β-sheet 3), which includes residues 40–48. The ion representing residues 43–48 showed the largest reduction in deuterium content for both the dCTP and dGTP binding events (Fig. 3C and Table 2). A modest decrease in the rate of HDX for residues 43–48 was also observed upon addition of all four dNTPs. The decreased amplitude of deuterium incorporation in the presence of dCTP is consistent with the results obtained for residues 48–63 and may represent more stable interactions between β-sheet 3 and αB-loop-αC when the nascent base pair is dCTP-dG (Fig. 3D).

Another ion that exhibited decreased rapid exchange upon addition of dCTP was identified as a peptide containing residues 151–170, which comprise the F-helix in the Dpo4 palm domain. The rate constant that defines the first exponential phase of HDX for the F-helix decreased ~3-fold upon addition of dCTP, indicating that conformational flexibility in this region is diminished during formation of the nascent base pair. However, the addition of other dNTPs also elicited a similar change in HDX rate, indicating that the decreased flexibility observed in the F-helix is a general phenomenon associated with formation of a ternary complex but one that is insensitive to nucleotide identity (Fig. 4).

Two peptides in the thumb domain revealed quite unique HDX kinetics during the binding of dNTPs. A peptide containing residues 180–202, which comprises α-helix H in the thumb, displayed increased HDX in the rapid exchange map upon addition of dCTP. Inspection of the HDX kinetic profiles revealed that the H-helix exhibits more stable and/or numerous hydrogen bonding interactions upon binding to DNA/Mg2+ and either dATP or dTTP (Fig. 5A and Table 2). The decrease in the rate of exchange was lost upon addition of either dCTP or (to a lesser extent) dGTP (Fig. 5A and Table 2). The K-helix, which is in close proximity to the H-helix in the Dpo4 structure, also exhibited an increase in HDX when dCTP was present, relative to was observed with only DNA and Mg2+ (Fig. 5B and Table 2). These two helices in the thumb domain appeared to be sensitive to the identity of the incoming nucleotide, and their importance regarding polymerase fidelity and kinetics is discussed below.

**TABLE 1**

Peptide sequences that exhibit changes in the rapid phase of HDX upon addition of primer/template DNA, MgCl2, and/or dCTP

| Sequence       | Residues | Fraction of amide hydrogens exchanged in 15 s: | Dpo4         | +DNA/Mg2+ | +DNA/Mg2+ dCTP |
|----------------|----------|-----------------------------------------------|-------------|-----------|-----------------|
| VFSGRF         | 32–37    | 0.30                                          | 0.11*       | 0.12      |
| VATANY         | 43–48    | 0.19                                          | 0.21        | 0.08      |
| YEARKFGVKAGIPVE| 48–63    | 0.19                                          | 0.14        | 0.08      |
| VYLMKBEYQQYS   | 72–85    | 0.22                                          | 0.04        | 0.07      |
| VYLMKBEYQQYVS  | 72–86    | 0.17                                          | 0.10        | 0.11      |
| VYQQVSSRIMLLR  | 80–93    | 0.38                                          | 0.28        | 0.21      |
| AKIADMAKPGIKVIDDEE| 151–170 | 0.12                                          | 0.08        | 0.01      |
| IADVPGIGNITAEKLKLQGINKL| 180–202 | 0.19                                          | 0.08        | 0.14      |
| FDKKGMRG      | 210–218  | 0.12                                          | 0.04        | 0.03      |
| GMIDAEAKKYLISLA| 215–229 | 0.24                                          | 0.17        | 0.26      |
| ARDEYNEPIRT   | 229–239  | 0.2                                           | 0.32        | 0.14      |
| SYKLEGIRPIAHLVVA| 272–288 | 0.13                                          | 0.03        | 0.02      |
| VTEL0          | 289–293  | 0.15                                          | 0.08        | 0.07      |

*a Boldface type indicates peptides that exhibited >5% change in deuterium content upon addition of the indicated substrates.

**FIGURE 2. Structural overview of rapid phase of HDX for Dpo4.** Dpo4 is shown in schematic form (cyan for regions analyzed by HDX, gray for regions not analyzed by HDX; pdb code 2j6t (13)). The changes in rapid HDX >5% of the possible amount for each peptide are shown in either red (decreased exchange) or green (increased exchange).
Dpo4 Conformational Dynamics Using H-D Exchange

The kinetics of HDX in the finger domain is sensitive to the identity of the incoming dNTP. A, the kinetic profile for HDX in residues 48–63 is plotted for Dpo4 alone (black circles), +DNA/Mg$^{2+}$ (blue squares), +DNA/Mg$^{2+}$/dCTP (red circles), +DNA/Mg$^{2+}$/dTTP (purple diamonds), +DNA/Mg$^{2+}$/dATP (green triangles), and +DNA/Mg$^{2+}$/dGTP (orange inverted triangles). The results are averages of two data sets. B, the kinetic profile for HDX in residues 49–63 is plotted for Dpo4 alone (black circles), +DNA/Mg$^{2+}$ (blue squares), +DNA/Mg$^{2+}$/dCTP (red circles), +DNA/Mg$^{2+}$/dATP (green triangles), and +DNA/Mg$^{2+}$/dGTP (orange inverted triangles). The observed rate constants are listed in Table 2. D, the structural orientation of the loop region (residues 48–63) of Dpo4 in the catalytic cycle.

The HDX-MS results obtained here provide evidence for two structural changes that are important for Dpo4 fidelity. Upon binding to an incoming dNTP the loop region between α-helices B and C appears to respond in a manner that stabilizes the hydrogen bonding patterns by decreasing structural fluctuations in the finger domain (Fig. 3). The flexibility of the loop region inferred from changes in the HDX rate is decreased most substantially during the formation of a Watson-Crick base pair in the pol active site. Given the fact that substantial changes in the finger domain are not observed in the crystal structures of Dpo4, it would seem possible that the decrease in the amount of deuterium observed in the loop region is simply due to solvent exclusion by dNTP binding and not a conformational change per se. In general terms, x-ray crystal structures are thought to represent the most thermodynamically stable conformation of a molecule under a given set of conditions (i.e. an endpoint). HDX methods report on regions of the protein that are undergoing structural changes, primarily those associated with breaking and/or formation of hydrogen bonds in the amide backbone. Our data support the idea that there is structural flexibility in the finger domain that is not reported by the crystal structures. As discussed under “Results,” HDX rates for a given peptide are not as sensitive to solvent exposure as might be expected on an intuitive level (31), which would preclude changes due to solvent exclusion upon dNTP binding. Moreover, the contacts observed between the protein and incoming dNTP in the loop region are mediated entirely by the amino acid side chains and not main-chain atoms. HDX analysis only

### Table 2: HDX kinetics for selected ions

| Residues | Substrates | $k_1$ | $k_2$ | $k_3$ | $k_4$ |
|----------|------------|-------|-------|-------|-------|
| 42–48    | DNA/Mg$^{2+}$ | 0.21 | 0.77 | 0.80 | 0.22 |
| 42–48    | DNA/Mg$^{2+}$/dCTP | 0.27 | 0.78 | 0.80 | 0.22 |
| 42–48    | DNA/Mg$^{2+}$/dATP | 0.27 | 0.78 | 0.80 | 0.22 |
| 42–48    | DNA/Mg$^{2+}$/dGTP | 0.27 | 0.78 | 0.80 | 0.22 |
| 49–63    | DNA/Mg$^{2+}$ | 0.21 | 0.77 | 0.80 | 0.22 |
| 49–63    | DNA/Mg$^{2+}$/dCTP | 0.27 | 0.78 | 0.80 | 0.22 |
| 49–63    | DNA/Mg$^{2+}$/dATP | 0.27 | 0.78 | 0.80 | 0.22 |
| 49–63    | DNA/Mg$^{2+}$/dGTP | 0.27 | 0.78 | 0.80 | 0.22 |
| 151–170  | DNA/Mg$^{2+}$ | 0.21 | 0.77 | 0.80 | 0.22 |
| 151–170  | DNA/Mg$^{2+}$/dCTP | 0.27 | 0.78 | 0.80 | 0.22 |
| 151–170  | DNA/Mg$^{2+}$/dATP | 0.27 | 0.78 | 0.80 | 0.22 |
| 151–170  | DNA/Mg$^{2+}$/dGTP | 0.27 | 0.78 | 0.80 | 0.22 |
| 180–202  | DNA/Mg$^{2+}$ | 0.21 | 0.77 | 0.80 | 0.22 |
| 180–202  | DNA/Mg$^{2+}$/dCTP | 0.27 | 0.78 | 0.80 | 0.22 |
| 180–202  | DNA/Mg$^{2+}$/dATP | 0.27 | 0.78 | 0.80 | 0.22 |
| 180–202  | DNA/Mg$^{2+}$/dGTP | 0.27 | 0.78 | 0.80 | 0.22 |
| 215–229  | DNA/Mg$^{2+}$ | 0.21 | 0.77 | 0.80 | 0.22 |
| 215–229  | DNA/Mg$^{2+}$/dCTP | 0.27 | 0.78 | 0.80 | 0.22 |
| 215–229  | DNA/Mg$^{2+}$/dATP | 0.27 | 0.78 | 0.80 | 0.22 |
| 215–229  | DNA/Mg$^{2+}$/dGTP | 0.27 | 0.78 | 0.80 | 0.22 |
Dpo4 Conformational Dynamics Using H-D Exchange

The evidence supporting an important role for rotation of the primer/template DNA during ternary complex formation is not confined to Dpo4. A similar conclusion was derived from the crystal structure of yeast pol η bound to cisplatin-containing DNA (35). In the pol η structure, two distinct primer/template DNA binding orientations were observed in the asymmetric unit. These modes were termed pre-elongation and elongation complexes. The general conclusion from comparing pre-elongation and elongation modes was that a rotation of the DNA was associated with the induced-fit mechanism of action. An overlay of the pre-elongation and elongation complexes does suggest that the thumb and little finger domains shift their orientation when the DNA rotates, which again highlights the importance of translocating the DNA into an active conformation during nucleotide selection by Y-family DNA polymerases.

Further evidence supporting an active role for the thumb domain during dNTP selection can be found in recent work from our group that used tryptophan fluorescence to measure the kinetics of conformational changes during Dpo4 bypass of the major oxidative DNA lesion 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxoG) reported on translocation mechanics by comparing pre-insertion binary, insertion ternary, and post-insertion binary complexes (19). The authors of the translocation study note that the contacts between the thumb domain (helices H, K, and J) and the primer/template DNA remain the same when comparing binary and ternary complexes. The model derived from the crystal structures proposes that the finger, palm, and little finger domains all shift forward by one nucleotide during binding of dCTP. However, the authors superimposed the binary and ternary DNA structures to draw their conclusions regarding domain rearrangements. A more accurate way to compare the structures would involve superimposition of the active site of the binary and ternary complexes (i.e. the palm domain) and searching for structural changes, because the DNA will be required to move relative to the catalytic site when the next dNTP binds. If the palm domains of the pre-insertion binary and insertion ternary complexes are superimposed, then comparison of the crystal structures reveals that the thumb domain changes its position relative to the finger, palm, and little finger domains when an accurate ternary complex is formed and the primer/template DNA shifts back to accommodate the incoming dNTP (Fig. 5C). We should note that the HDX results reported here do not directly measure the direction or magnitude of changes suggested by the superimposition of crystallographic results (Fig. 5C).

However, based on the crystallographic results, the conformational change in the thumb domain reported by HDX is most likely a result of the DNA rotating “back” out of the active site to accommodate the nascent base pair (Fig. 5C). The contacts remain fixed, as observed in the crystal structures, but the exposure to solvent and equilibrium between binary/ternary DNA positions shifts toward ternary, which results in increased HDX in the thumb domain. The other domains still translocate forward by one nucleotide, as suggested in the crystallographic study, but it is because the DNA is rotated in the Dpo4 active site by one nucleotide. It is effectively the thumb domain combined with a fixation of the dNTP by the finger domain that serves to promote a catalytically active complex.

The evidence supporting an important role for rotation of the primer/template DNA during ternary complex formation is not confined to Dpo4. A similar conclusion was derived from the crystal structure of yeast pol η bound to cisplatin-containing DNA (35). In the pol η structure, two distinct primer/template DNA binding orientations were observed in the asymmetric unit. These modes were termed pre-elongation and elongation complexes. The general conclusion from comparing pre-elongation and elongation modes was that a rotation of the DNA was associated with the induced-fit mechanism of action. An overlay of the pre-elongation and elongation complexes does suggest that the thumb and little finger domains shift their orientation when the DNA rotates, which again highlights the importance of translocating the DNA into an active conformation during nucleotide selection by Y-family DNA polymerases.

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FIGURE 5. Changes in HDX kinetics for the thumb domain are sensitive to the identity of the incoming dNTP. A, the kinetic profile for HDX in residues 180–202 is plotted for Dpo4 alone (black circles), +DNA/Mg\(^2^+\) (blue squares), +DNA/Mg\(^2^+\)/dCTP (red circles), +DNA/Mg\(^2^+\)/dTTP (purple diamonds), +DNA/Mg\(^2^+\)/dATP (green triangles), and +DNA/Mg\(^2^+\)/dGTP (orange inverted triangles). The results are averages of two data sets. B, the kinetic profile for HDX in residues 215–229 is plotted for Dpo4 alone (black circles), +DNA/Mg\(^2^+\) (blue squares), +DNA/Mg\(^2^+\)/dTTP (red circles), +DNA/Mg\(^2^+\)/dTTP (purple diamonds), +DNA/Mg\(^2^+\)/dATP (green triangles), and +DNA/Mg\(^2^+\)/dGTP (orange inverted triangles). The results are averages of two data sets. The HDX rate-constants for A and B are listed in Table 2. C, a superimposition of the palm domain for pre-insertion binary (gray, pdb ID code 2asj (19)) and insertion ternary (green, pdb ID code 2asd (19)) is shown for the region of the thumb domain that exhibits HDX kinetics sensitive to the identity of the incoming dNTP.

catalysis (25). The wild-type Dpo4 sequence does not contain any endogenous tryptophan residues. The Trp mutant used to analyze conformational kinetics was Dpo4-T239W (25). Importantly, Thr-239 is located in the linker region adjacent to the thumb domain. The primer/template DNA sequence used in the fluorescence study is identical to the one used here. Changes in fluorescence were only observed when the incoming dNTP formed either a “Type I” Watson-Crick base pair or a “Type II” –1 frameshift deletion and not when dNTPs forming mispairing events (i.e. dATP or dTTP) were included in the reaction. In reactions with dideoxy-terminated primers the fluorescence change was a monophasic increase. The maximal extrapolated rate-constant defining the fluorescent increase was \(\sim 35 \text{ s}^{-1}\) (25). When phosphodiester bond formation could occur (i.e. 3’-hydroxyl-containing primer) a second, slower phase of decreasing fluorescence (\(k_{\text{obs}} = \sim 1.5 \text{ s}^{-1}\)) was observed after the fast increase. The slow decay appears to be first-order in nature because the rate was insensitive to dCTP concentration. The slow fluorescent decay was attributed to a “relaxation” event following phosphodiester bond formation. By measuring the kinetics of pyrophosphate release the relaxation event was determined to occur following pyrophosphate release. Such a model is consistent with the HDX results reported here. In the absence of phosphodiester bond formation the relaxation event in the thumb domain (Fig. 5) did not occur for either the accurate ternary complex (dCTP) or a Type II complex (dGTP), consistent with the increased rate of HDX observed for this region of Dpo4.

There are dynamic interactions between Dpo4, DNA, dNTPs, and metal ions in addition to the structural rearrangements noted above. As mentioned previously, upon binding to DNA the little finger domain apparently undergoes a dramatic conformational change that includes a large rotation and translocation through space (20). The binding of dNTP and metal ions is thought to be rapid (\(k_{\text{forward}} \sim 10^8 \text{ M}^{-1} \text{ s}^{-1}\)), with the rate of dNTP dissociation providing an initial level of nucleotide discrimination that is manifest in the apparent equilibrium binding constant (\(K_D\)dNTP). Based on data reported here and elsewhere...
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the loop region (residues 48–63), αB, αC, and β3 in the finger domain (Fig. 3D).

Dpo4 is classified in the DinB subfamily of the Y-family DNA polymerases (9). It is of interest to consider the implications of change observed in the finger domain (residues 48–63) of Dpo4 to the human DinB homologue pol κ. There is substantial evidence that the Y-family DNA polymerase κ is important during bypass of bulky minor groove adducts, especially products arising from exposure to exogenous polycyclic aromatic hydrocarbons (e.g. benzo[α]pyrene) and/or endogenous products of steroid biosynthesis (36–40). pol κ is structurally similar to Dpo4 in overall domain topology with one major exception. pol κ possesses an N-terminal extension termed the N-clasp, which forms two α-helices that were shown to encircle the DNA in a crystal structure of the pol κ ternary complex (10).

The first helix in the N-clasp sits directly atop the finger domain of pol κ, and the second helix traverses the DNA at a 45° angle before connecting with and packing against the thumb domain. The N-clasp is essential for pol κ function, because deletion of the first 68 residues results in a dramatic loss of activity, including a substantial decrease in the binding affinity for DNA (10). Given the fact that the N-clasp contacts both domains that appear to be central to structural rearrangements during Dpo4 nucleotide selection, it will be of great interest to investigate whether pol κ exhibits similar dynamics. A recent study from our group investigated pol κ-catalyzed bypass of 8-oxoG,4 and the results obtained there are consistent with more restrained conformational dynamics for pol κ. Specifically, we found that the error-prone nature of pol κ bypass is determined by the stabilization of syn-oriented 8-oxoG by residues in the finger domain of pol κ and a lack of electrostatic interactions needed to stabilize the anti conformation of 8-oxoG. The 8-oxoG adduct is thermodynamically more stable in the syn orientation (41), and its mutagenicity stems from the ability of many polymerases to form a Hoogsteen base pair between dATP and anti to stabilize the

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