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Identification of an Unfolding Intermediate for a DNA Lesion Bypass Polymerase

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Abstract

Sulfolobus solfataricus DNA Polymerase IV (Dpo4), a prototype Y-family DNA polymerase, has been well characterized biochemically and biophysically at 37 °C or lower temperatures. However, the physiological temperature of the hyperthermophile *S. solfataricus* is approximately 80 °C. With such a large discrepancy in temperature, the *in vivo* relevance of these *in vitro* studies of Dpo4 has been questioned. Here, we employed circular dichroism spectroscopy and fluorescence-based thermal scanning to investigate the secondary structural changes of Dpo4 over a temperature range from 26 to 119 °C. Dpo4 was shown to display a high melting temperature characteristic of hyperthermophiles. Unexpectedly, the Little Finger domain of Dpo4, which is only found in the Y-family DNA polymerases, was shown to be more thermostable than the polymerase core. More interestingly, Dpo4 exhibited a three-state cooperative unfolding profile with an unfolding intermediate. The linker region between the Little Finger and Thumb domains of Dpo4 was found to be a source of structural instability. Through site-directed mutagenesis, the interactions between the residues in the linker region and the Palm domain were identified to play a critical role in the formation of the unfolding intermediate. Notably, the secondary structure of Dpo4 was not altered when the temperature was increased from 26 to 87.5 °C. Thus, in addition to providing structural insights into the thermal stability and an unfolding intermediate of Dpo4, our work also validated the relevance of the *in vitro* studies of Dpo4 performed at temperatures significantly lower than 80 °C.

INTRODUCTION

Y-family DNA polymerases are prevalent in all three domains of life and catalyze DNA synthesis with low fidelity and poor processivity. Importantly, these enzymes, unlike replicative DNA polymerases, are capable of bypassing a variety of DNA lesions due to their flexible and solvent-accessible active sites.^{1–7} As a model and lone Y-family DNA

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SUPPORTING INFORMATION AVAILABLE

The CD spectra of many Dpo4 mutants, thermal denaturation in the presence of guanidine hydrochloride, amino acid sequence alignment of linker regions for different Y-family DNA polymerases and the table on proposed interactions are available as supporting information. These materials are free of charge via the internet at <http://pub.acs.org>.

polymerase in *Sulfolobus solfataricus*, DNA polymerase IV (Dpo4) has been extensively studied biochemically and biophysically. *S. solfataricus* is a hyperthermophile that thrives in an extreme environment of 80 °C and pH 2 to 3.⁸ The genomic DNA of *S. solfataricus* is likely to be damaged frequently and the lesion bypass pathway is one of the major mechanisms used by the archaeon to cope with DNA damage during replication and maintain an error rate that is lower than higher-order organisms.⁹ *In vitro*, Dpo4 has been shown to bypass various lesions including an abasic site,^{10–13} 7,8-dihydro-8-oxodeoxyguanosine,^{14–16} 1,*N*²-etheno(ε)deoxyguanosine,¹⁷ *cis-syn* cyclobutane pyrimidine thymine-thymine dimer,^{18–20} 1,2-cisplatinated deoxyguanosine,^{18, 21, 22} deoxyadenosine with a benzo[*a*]pyrene diol epoxide adduct,²³ *N*-(deoxyguanosin-8-yl)-1-aminopyrene,²⁴ and *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene.¹⁸ Interestingly, a *dpo4* knockout strain of *S. solfataricus* has been shown to have significantly inhibited growth compared to the wild-type organism in the presence of cisplatin,²¹ a DNA damaging anti-cancer drug. This study suggests that Y-family DNA polymerases play a major role in the bypass of cisplatin-DNA adducts *in vivo*.

Although functioning at 80 °C *in vivo*, Dpo4 can catalyze nucleotide incorporation into undamaged DNA at temperatures as low as 2 °C with a fidelity in the range of 10^{–3} to 10^{–4} as demonstrated by our pre-steady state kinetic analyses.^{25–27} Moreover, the fidelity remains relatively unchanged over a temperature range of 26 to 56 °C.²⁷ Notably, approximately 90% of the polymerase activity of Dpo4 remains after incubation at 95 °C for 5 min.¹⁸ These temperature-dependent studies suggest that no substantial structural changes occur for Dpo4 over a broad range of temperatures. However, this hypothesis has never been directly investigated by structural analysis. Interestingly, crystallographic and tryptophan fluorescence studies of Dpo4 have revealed that upon DNA binding, Dpo4 undergoes a large conformational change upon Dpo4•DNA binary complex formation with a 131° rotation of the Little Finger (LF) domain relative to the polymerase core (Core) which is composed of the Finger, Palm, and Thumb domains (Figure 1).²⁸ Furthermore, when bound to *S. solfataricus* proliferating cell nuclear antigen (PCNA), a processivity factor, the position of the LF relative to the Core is distinct from that observed in both the apo- and DNA-bound states (Figure 1).²⁹ The observation of these differing conformations leads to the identification of a flexible “hinge” in the linker region connecting the LF to the Thumb domain.²⁹ During the binding and incorporation of a correct incoming nucleotide into either undamaged³⁰ or damaged³¹ DNA, Dpo4 has also been shown to undergo global conformational changes based on our stopped-flow FRET studies.

In this study, we employed circular dichroism (CD) spectroscopy and fluorescence-based thermal scanning (FTS) to investigate the structural stability and the unfolding process of Dpo4. Our results demonstrate that Dpo4 unfolded in a three-state, cooperative manner with a melting temperature (*T*_m) well above the physiological temperature of *S. solfataricus*. The origin of the unfolding intermediate of Dpo4 was identified via thermal denaturation analysis of multiple mutants of Dpo4.

MATERIAL AND METHODS

Protein Purification

Wild-type *S. solfataricus* Dpo4 fused to a C-terminal His₆-tag was overexpressed in *E. coli* and purified as previously described.²⁶ The following truncation mutants of Dpo4 were generated by molecular subcloning, expressed in *E. coli*, and purified using the same protocol for wt Dpo4:²⁶ the Core fragment (residues 1–230), the LF⁺ fragment (amino acid residues 231–352), and the LF fragment (residues 246–352). The following full-length Dpo4 mutants (Table 1) were generated by site-directed mutagenesis, expressed in *E. coli*, and purified as wt Dpo4: the All-Gly linker mutant, the R/K-to-A mutant, the R/K-to-D mutant,

the E100A mutant, the K148A mutant, the E100A/K148A mutant, the E100A/E235A/R240A mutant, the K148A/E235A/R240A mutant, the E100A/K148A/E235A/R240A mutant, the P236A mutant, the R240 mutant, the E235A/R240A mutant. Prior to experiments, these purified proteins were exchanged with degassed buffers and the final concentrations were determined spectrophotometrically at 280 nm using the following extinction coefficients: 24,058 M⁻¹cm⁻¹ for wt Dpo4 and its full-length mutants except the All-Gly linker mutant; 22,568 M⁻¹cm⁻¹ for the All-Gly linker mutant, 16,608 M⁻¹cm⁻¹ for the Core fragment, 7,450 M⁻¹cm⁻¹ for the LF⁺ fragment, and 5,960 M⁻¹cm⁻¹ for the LF fragment.

Circular Dichroism Spectroscopy

CD spectra were obtained using an AVIV CD spectrometer model 62A DS. Unless specified, CD spectra were acquired in a 1 mm quartz cuvette at 37 °C over a wavelength range typically from 200 nm to 270 nm using an iteration of 1 nm and 5 s signal averaging at each wavelength. Protein samples with final concentration of approximately 1 mg*mL⁻¹ were dissolved into degassed buffer A (25 mM Na₃PO₄, pH 7.5, 50 mM NaCl, and 5 mM MgCl₂) and filtered with a 0.45 µm membrane to remove residual aggregation. The CD spectrum of the buffer alone was obtained and subtracted from each sample CD spectra. Each ellipticity value was converted to a corresponding molar ellipticity value (deg*cm²*dmol⁻¹), and was then plotted as a function of wavelength.

CD spectroscopic-based thermal denaturation plots were acquired over a temperature range from 26 to 119 °C using an iteration of 1.5 °C and a 1 mm path length quartz cuvette. The cuvette was stoppered to ensure that there was no loss in total sample volume during thermal denaturation. Molar ellipticity values (θ_m) were plotted as a function of temperature (°C) at a fixed wavelength of 222 nm or 209 nm. The thermal denaturation data were used to calculate the apparent equilibrium constant of unfolding at temperature T (K_T) in the following equation:

$$K_T = [\theta_{m, \text{obs}} - (y_f + m_f * T)] / [(y_u + m_u * T) - \theta_{m, \text{obs}}]$$

where $\theta_{m, \text{obs}}$ is the observed θ_m at a fixed wavelength, T is the temperature (Kelvin), y_f and m_f are the y-intercept and slope of the linear baseline before the unfolding transition, respectively, and y_u and m_u are the y-intercept and slope of the linear baseline after the unfolding transition, respectively.³² Based on the van't Hoff equation, apparent ΔH_m and ΔS_m were derived from the plot of $\ln K_T$ versus $1/T$.

For thermal denaturation in the presence of 50 mM guanidine hydrochloride, each protein sample was prepared in degassed buffer A as described above. First, a CD spectrum was obtained for the sample. Then, concentrated guanidine hydrochloride in buffer A was added to the protein sample. After incubation of the mixture for 10 min at 37 °C, another CD spectrum was obtained to ensure the protein was still folded. Finally, the thermal denaturation plot of the same sample of protein was acquired as described above.

Fluorescence-Based Thermal Scanning (FTS) Assay

The FTS assays were performed by using an iCycler iQ Real-Time Detection System (Bio-Rad) and following previously published protocols.^{33, 34} Solutions of 2 µL of SYPRO® Orange (30X final concentration, Sigma-Aldrich) and 18 µL of a protein sample (approximately 1 mg*mL⁻¹) in buffer A were added to each well of a 96-well thin-wall PCR plate (Bio-Rad). Then, each well was sealed with iCycler optical quality sealing tape (Bio-Rad). After sealing, the plates were heated from 25 to 94.8 °C with 0.2 °C increments per 12

s and the thermal denaturation data were acquired by monitoring the fluorescence intensities at 575 nm upon excitation at 490 nm.

RESULTS

Determination of Secondary Structure Stability

The CD spectra of wild-type (wt) Dpo4 and its mutants (Figure 2 and Table 1) were obtained in the far-UV region (Figure 3 and Supplementary Figure 1) to identify any significant changes in secondary structure under non-denaturing conditions. Notably, the CD spectra indicated that all Dpo4 mutants were folded at 37 °C. In the CD spectrum of wt Dpo4 (Figure 3), strong negative molar ellipticity (θ_m) observed at 209 and 222 nm indicated that Dpo4 possess a substantial amount of α -helical content.³⁵ All full-length Dpo4 constructs containing mutations in the linker region (Table 1) had CD spectra nearly identical to that of wt Dpo4 (Figure 3 and Supplementary Figure 1), which suggests that the linker region had little effect on the secondary structure contents of Dpo4 at 37 °C. Interestingly, while the CD spectra of wt Dpo4 and the Core fragment (Figure 2) were similar in shape (Figure 3), the Core exhibited significantly stronger negative molar ellipticity at 222 and 209 nm owing to an increase in the relative amount of α -helical content. In contrast, the CD spectrum of the LF⁺ fragment (Figure 2) did not reveal two distinct peaks at 222 and 209 nm, suggesting that α -helices were not the dominant secondary structure in the LF⁺. Interestingly, in the absence of the linker region, the LF fragment (Figure 2) had a fairly low solubility in salt concentrations less than 400 mM NaCl at 23 °C. Nevertheless, the far-UV CD spectra of the LF at 37 °C in low and high salt buffers were very similar to the CD spectrum of the LF⁺, but displayed more negative θ_m (Figure 3 and Supplementary Figure 2a), indicating that the linker region did not contain any α -helices.

Monitoring Thermal Denaturation of Dpo4 by CD Spectroscopy

In order to monitor potential changes in secondary structure upon heating, a series of CD spectra were obtained for a single sample of wt Dpo4 at various temperatures between 38 and 100 °C. The sample was equilibrated for 5 min at each specific temperature before the corresponding CD spectrum was acquired. As shown in Supplementary Figure 3, only the amplitude of the molar ellipticity, not the shape of the CD spectrum, changed at higher temperatures, indicating a decrease in the total amount of folded protein, rather than a change in the secondary structural composition of Dpo4. Markedly, the most significant changes in molar ellipticity occurred upon increases in temperature from 80 to 90 °C and from 95 to 100 °C. This observation suggests the presence of two potential events in the thermal denaturation pathway of Dpo4.

To further investigate the unfolding events, we monitored thermal denaturation of Dpo4 via CD spectroscopy at a fixed wavelength of 222 nm. As shown in Figure 4, wt Dpo4 exhibited a three-state cooperative unfolding profile, with an unfolding intermediate clearly existing between the native and denatured states. The native state of wt Dpo4 persisted from 26 to 87.5 °C and the unfolding intermediate was observed between 92 and 96.5 °C. Starting at 110 °C, the molar ellipticity approached its highest value and did not change significantly in the temperature range of 110 to 119 °C. At 119 °C, the temperature limit of the CD spectrometer, approximately 0.3 percent of wt Dpo4 retained secondary structure as calculated from the ratio of the θ_m value at 119 °C to that at 26 °C. Notably, after heating to 100 °C or above, wt Dpo4 did not refold to its native state when temperature was decreased to ambient conditions since the θ_m value did not change significantly (data not shown). In addition, most Dpo4 was in the aggregated form within the sealed cuvette after thermal denaturation at 119 °C, indicating that the observed change in molar ellipticity in Figure 4

was due to protein unfolding, rather than a change in sample volume. Similar results were obtained by monitoring the thermal denaturation of wt Dpo4 via CD spectroscopy at a fixed wavelength of 209 nm (comparing Figure 4 and Supplementary Figure 4).

Based on the thermal denaturation plot in Figure 4, K_T , the unfolding equilibrium constant at temperature T (see Material and Methods), was calculated for wt Dpo4. Each plot of $\ln(K_T)$ versus $1/T$ for each unfolding transition or overall unfolding (Figure 5a) was fit to van't Hoff equation to yield a slope and an intercept which were used to calculate the change in melting enthalpy (ΔH_m) and the change in melting entropy (ΔS_m) (Table 2). The plot of the free energy for unfolding (ΔG_m) versus temperature T (Figure 5b) was fit to Gibbs energy equation. The resulting intercept on the X-axis, where ΔG_m was equal to zero, represents the overall melting temperature (T_m) for wt Dpo4, for the native state, or for the unfolding intermediate (Table 2). Notably, the data for the overall unfolding of wt Dpo4 were not fit well to linear van't Hoff equation or Gibbs energy equation (Figure 5), confirming that there were more than one transition during the unfolding of wt Dpo4 (Figure 4). Due to the irreversible unfolding of wt Dpo4, the corresponding thermodynamic parameters in Table 2 should be considered as apparent values.³⁶

Probing Thermal Denaturation of Dpo4 Fragments by CD Spectroscopy

The observed unfolding intermediate could be made up of an unfolded LF domain with a folded Core or vice versa. To examine these possibilities, thermal denaturation of the LF, LF⁺ and Core fragments (Figure 2) was also separately monitored via CD spectroscopy at a fixed wavelength of 222 nm. As shown in Figure 4, the Dpo4 truncation mutants exhibited cooperative unfolding with T_m values higher than 80 °C (Table 2). Surprisingly, both the Core and LF fragments displayed thermal denaturation curves without any unfolding intermediate and were more thermally stable than wt Dpo4 (Figure 4 and Table 2). In fact, the LF fragment did not start unfolding until 95 °C and was not completely unfolded at 119 °C (Figure 4). When the salt concentration of the buffer was increased from 50 to 400 mM in order to increase the solubility of the LF, unfolding was not observed even at 119 °C (Supplementary Figure 2b). Since there was no unfolded state observed for the LF, the apparent unfolding thermodynamic parameters could not be calculated.

Surprisingly, an unfolding intermediate was also observed for the LF⁺ fragment (Table 1) and the presence of the linker region decreased the thermal stability of the LF domain (Table 2 and Figure 4). It is possible that the linker region, with its high density of charged residues (Table 1), was able to destabilize the LF domain through interactions that are not possible in wt Dpo4 when the linker region is tethered to the Thumb domain. Collectively, these results suggest that the observation of an unfolding intermediate was likely dependent on the disruption of the interactions between the linker region and the rest of Dpo4, rather than independent unfolding of the domains of Dpo4 at discrete temperatures. Furthermore, the greater overall T_m of the individual truncation mutants compared to wt Dpo4 suggests that the interactions, which gave rise to the unfolding intermediate, acted to reduce the overall thermal stability of Dpo4. Based on the ratio of the θ_m value at 119 °C to that at 26 °C, approximately 3.9% of the Core, 7.9% of the LF⁺, and 35.7% of the LF fragment remained folded at 119 °C, compared to only 0.3% for wt Dpo4 (Figure 4). In comparison, the order of thermal stability of Dpo4 fragments is wt Dpo4 < LF⁺ < Core < LF based on the overall T_m values in Table 2.

Monitoring Thermal Denaturation of Dpo4 Fragments by FTS

To confirm relative thermal stability of Dpo4 fragments derived from CD studies, we monitored thermal denaturation of wt Dpo4 and the LF and the LF⁺ fragments using FTS assays (Materials and Methods). The Core fragment was not stable in solution under the

conditions required for FTS and therefore could not be studied with this technique. During an FTS assay, the fluorescent quantum yield of a hydrophobic dye (SYPRO® Orange in this study) increases as the dye binds to the hydrophobic interior of a protein which gradually becomes more accessible during an unfolding process. After the fluorescence emission of the dye reaches its maximum, it will decrease if the sample is continuously heated, likely due to the precipitation of the dye with the denatured protein. In Figure 6, the FTS plot of wt Dpo4 displays a maximum fluorescence at 86.2 °C while no maximum was observed in the FTS plots of the LF⁺ and LF fragments even when the temperature limit of the instrument (94.8 °C) was reached. Notably, the unfolding intermediate could not be observed using this FTS assay because the instrument was unable to reach temperatures required to fully denature wt Dpo4, as seen in the CD spectroscopy assay (Figure 4). Nevertheless, the initial unfolding temperatures from the FTS analysis (Figure 6) confirmed the above order of thermal stability: wt Dpo4 < LF⁺ < LF.

Determination of the Origin of the Unfolding Intermediate

In order to identify the physical nature of the unfolding intermediate of Dpo4, a series of mutations were introduced into the linker region. We first investigated the thermal denaturation of a Dpo4 mutant (All-Gly linker) in which all linker region amino acid residues were substituted with glycine (Table 1). As observed with the Core fragment, the All-Gly linker mutant exhibited a higher overall T_m than wt Dpo4 (Table 2) and possessed no unfolding intermediate (Figure 7a). These results confirm the above conclusion that the linker region was involved in the destabilizing interactions observed in wt Dpo4 and in the formation of the unfolding intermediate. In comparison, a three-state thermal denaturation profile with an unfolding intermediate (Figure 7a) was observed with another full-length Dpo4 mutant (P236A) in which the single proline residue in the linker region was substituted with alanine (Table 1). However, relative to wt Dpo4, the native state of P236A unfolded more gradually, starting at a lower temperature. Moreover, more P236A remained folded when the unfolding intermediate was formed, as indicated by its lower θ_m values than those of wt Dpo4.

To determine if any ionic interactions between the linker region and the Core and/or LF domain played a role in the formation of the unfolding intermediate, we performed thermal denaturation assays on wt Dpo4 and the P236A mutant in the presence of 50 mM guanidine hydrochloride. At this low concentration, the chaotropic effect of the guanidine hydrochloride was insufficient to denature the proteins, as shown in Supplementary Figure 5a, but did remove the unfolding intermediates for both wt Dpo4 and the P236A mutant (Supplementary Figure 5b). These data suggest that the formation of the unfolding intermediates was likely influenced by ionic interactions involving the linker region. The apparent T_m values in the presence of 50 mM guanidine hydrochloride (Tables 2 and 3) were either similar (for P236A) or slightly higher (for wt Dpo4) than the corresponding values measured in the absence of this chaotropic salt, indicating that the overall protein thermal stability was not affected by the low concentration of guanidine hydrochloride.

Among the 14 amino acid residues in the linker of Dpo4, nine are charged (Table 1). To identify which of them participated in the ionic interactions with the domains of Dpo4, we created several point mutations within the linker region (Table 1) in order to disrupt these ionic contacts. In our first attempt, we replaced all positively charged linker residues with neutral (R/K-to-A) or negatively charged (R/K-to-D) residues (Table 1). As shown in Figure 7a, these mutations were sufficient to remove the observed unfolding intermediate from the thermal denaturation curves. Intriguingly, the R/K-to-D mutant was the most thermostable full-length Dpo4 mutant studied here, with an apparent T_m of 104.4 ± 0.4 °C (Table 4). These findings suggest that the positively charged amino acid residue(s) within the linker region played an important role in the formation of the unfolding intermediate (Figure 7a).

To further determine the identity of the charged linker residues, we inspected the structure of apo-Dpo4 (Figure 8a) and identified ionic interactions between amino acid residues in the Palm domain (E100 and K148) and the linker (E235 and R240), which could be responsible for the formation of the unfolding intermediate. Dpo4 mutants with combinations of these amino acids mutated to alanine were generated and subjected to thermal denaturation. Consistent with our predication, changing one (E100A, K148A, or R240A) or a few of these residues to alanine was sufficient to remove the unfolding intermediate (Figure 7b). Notably, all of these mutants except R240A had a lower T_m value (Table 4) than the overall T_m of wt Dpo4 (96 °C, Table 2).

DISCUSSION

Thermal Stability of the Secondary Structure of Dpo4 in Solution

Analysis of the apo-Dpo4 structure²⁸ shows that full-length Dpo4 and its Core and LF fragments in crystals have 48, 56, and 36%, respectively, of their amino acid residues in α -helices. In comparison, based on the molar ellipticity values at 222 nm in the CD spectra (Figure 3), the relative α -helical content follows the order of Core > wt Dpo4 > LF. Thus, the results derived from our solution-phase CD spectra agree well with the published structure of Dpo4 in solid phase.²⁸ More interestingly, all Dpo4 truncation and point mutants did not start to unfold until after 80 °C (Figures 4, 6 and 7) and the overall shape of the CD spectrum of wt Dpo4 remained constant over a wide temperature range (38–100 °C) (Supplementary Figure 3). Therefore, the overall secondary structure of Dpo4 at 37 °C or lower temperatures is very similar to that of Dpo4 at 80 °C, the physiological temperature of *S. solfataricus*. Accordingly, the biochemical and biophysical studies of Dpo4 performed at ambient temperatures, especially at 37 °C, due to instrument limitation are indeed structurally and biologically relevant. Consistently, our temperature-dependent kinetic studies of Dpo4³⁷ also suggest the characteristic activity of Dpo4 is not compromised at lower-than-physiological temperatures.

Unfolding Intermediate of Dpo4

The thermal denaturation plot of wt Dpo4 displayed a three-state cooperative unfolding profile with a distinct unfolding intermediate (Figure 4). Previous studies have demonstrated that similar observations of unfolding intermediates were due to the sequential melting of separate protein domains in esterase 2 from *Alicyclobacillus acidocaldarius* and human apolipoprotein E3, as determined through CD spectroscopic analyses of the combinations of different truncation mutants of the proteins.^{38, 39} However, this was not the case for Dpo4 as both the LF and Core domains were shown to have a higher thermal stability than the full-length protein (Table 2). Through the thermal denaturation studies of wt Dpo4 in the presence of 50 mM guanidine hydrochloride (Supplementary Figure 5) and the various linker region mutants of Dpo4 (Figure 7), we showed that this three-state unfolding behavior was instead due to ionic interactions between the linker region and the Palm domain observed in the apo-Dpo4 structure (Figure 8a).²⁸ Disruption of either of two critical ion pairs between the Palm domain (K148 and E100) and the linker region (E235 and R240) was sufficient to eliminate the unfolding intermediate (Figure 7b). Interestingly, the P236A mutation altered the thermodynamic unfolding properties, by lowering the structural stability of the native state and stabilizing the intermediate (Figure 7a). This substitution may have introduced additional conformational flexibility into the linker which destabilized the original ion pairs but did not completely prevent them from forming. Interestingly, the importance of these interactions is also emphasized by the fact that these charged residues are conserved in other Y-family DNA polymerases including *Escherichia coli* DinB, *S. acidocaldarius* Dbh, and human Y-family DNA polymerases (Supplementary Figure 6).^{4, 40–43}

In addition to the two aforementioned critical ion pairs, the large variations in the thermodynamic unfolding parameters for the linker region mutants (Tables 2 and 4) are suggestive of a more complex network of interactions involving the linker region amino acid residues. All mutations involving only the specific aforementioned ion pairs, except R240A, possessed a lower T_m than the overall T_m for wt Dpo4 while the broader linker region mutations (All-Gly linker, R/K-to-D, R/K-to-A, and P236A) displayed a higher thermal stability than Dpo4 (Tables 2 and 4). Inspection of the apo-Dpo4 crystal structure indicates that K148 and E100 also form a strong salt bridge with each other (Figure 8a), and therefore mutation of these residues may destabilize the Palm domain itself, leading to an overall lower thermal stability (Table 4). Similarly, as E235 and R240 are also involved in a charge-charge interaction with each other (Figure 8a), the E235A/R240A mutation, which also lowered the observed T_m (Table 4), may have acted synergistically to weaken the interaction between K148 and E100. Disruption of individual ionic interactions in ion pair networks involving multiple interacting residue pairs has been found to have similar destabilizing effects on other proteins including lambda repressor,⁴⁴ *Thermotoga maritima* D-glyceraldehyde-3-phosphate dehydrogenase,⁴⁵ and *Pyrococcus furiosus* glutamate dehydrogenase.⁴⁶ Notably, compared to the other point mutations, the R240A mutation may have been less disruptive to this ion pair network as the R240-E235 and R240-K148 ion pairs were longer range (*i.e.* 4 Å) than the E235-K148 or E100-K148 ion pairs (*i.e.* 2.6 Å) (Supplementary Table 1).

Furthermore, the unfolding of Dpo4 was also likely influenced by additional interactions between the Palm domain and linker region as well as interactions between neighboring amino acid residues in the linker region. Indeed, close inspection of the crystal structure of apo-Dpo4 reveals several hydrogen bonds between the Palm domain and linker region, *e.g.* the hydrogen bonding between the side chain oxygen of T239 and the backbone amide of I101, as well as hydrogen bonding and electrostatic interactions between neighboring amino acid residues in the linker region (Figure 8a and Supplementary Table 1). These contacts would be disrupted in the R/K-to-A, R/K-to-D and All-Gly linker mutants, but not in the mutants only affecting the ion pairs. The close proximity of the positively and negatively charged amino acid side chains in the linker region (E235, R238, R240, R242 and K243) likely imposed some additional structural restraints on the linker region (Figure 8a). On the basis of the T_m values (Tables 2 and 4), disruption of these additional interactions, as observed in the thermal denaturation curves of the R/K-to-A, R/K-to-D, and All-Gly linker mutants (Figure 7a, Tables 2 and 3) appear to have had an overall stabilizing effect on the full-length protein. In addition, the Core prevents the linker from improperly interacting with the LF domain as inferred from the thermal denaturation of the LF⁺ fragment (Figure 4).

Functional Implication of the Unfolding Intermediate of Dpo4

Interestingly, when Dpo4 is in the binary complex with DNA, the linker region adopts a more extended conformation and most of the hydrogen bonding and ion pair interactions involving the Palm domain and linker region are not possible (Figure 8c and Supplementary Table 1).²⁸ When in the PCNA-bound conformation, some of the interactions are lost while others are replaced (Figure 8b and Supplementary Table 1).²⁹ For example, the backbone amide of P236 and the backbone carbonyl oxygen of R238 in the linker region take the place of the side chains of other linker region residues E235 and R240 in interacting with the Palm residues E100 and K148 (Figure 8b). Despite their destabilizing effect to the native state of the apo-Dpo4 based on the T_m values (Tables 2 and 4), these interactions in the linker region and Palm domain likely have functional implications for Dpo4 as it transitions between the apo-, PCNA-bound, and DNA-bound states. Our preliminary DNA binding studies show that the All-Gly linker mutant has 250-fold weaker DNA binding affinity than wt Dpo4

(data not shown). It indicates that the residue composition of the linker region plays a critical role in DNA binding by Dpo4. While the linker region is likely somewhat flexible, the network of interactions shown in Figure 8 may act to limit the conformational space which the LF domain can occupy relative to the Core domain in order for Dpo4 to bind tightly to DNA and possibly PCNA. Importantly, our results show that these interactions were quite strong as they were not disrupted until wt Dpo4 was heated to ~92 °C, the temperature at which the unfolding intermediate was initially observed (Figure 4). There is thus a significant energetic cost in breaking these interactions as Dpo4 transitions between the apo-, PCNA-bound, and DNA-bound conformations. Notably, CD spectra recorded at temperatures where the intermediate was observed show no change in the overall secondary structure content of Dpo4 (Supplementary Figure 3), suggesting that the LF and Core domains both remained well folded in the intermediate state. As shown in Figure 9, the unfolding intermediate (I) observed in our thermal denaturation studies may represent a conformation similar to a high energy intermediate state (I') between the apo- and PCNA-bound conformations or between PCNA-bound and DNA-bound conformations, in which the LF domain can move freely relative to the Core. In this scenario, the interactions involving the linker and Palm domain residues would need to be broken in order for the LF to move relative to the Core when transitioning from the apo- to the PCNA-bound and then to the DNA-bound conformation (Figure 9). DNA lesion bypass, the *in vivo* function of Dpo4, involves polymerase switching between a Y-family DNA polymerase and a replicative DNA polymerase at the replication fork when a lesion is encountered.¹⁴ This process must be tightly regulated as Y-family polymerases are more error prone than replicative DNA polymerases when replicating undamaged DNA. Consequently, the interaction networks described in Figure 8 likely provide a mechanism for coordinating the movements of the LF domain which can contribute to the regulation of Dpo4 binding to the replication fork during DNA translesion synthesis.

Unfolding Order of Dpo4's Domains

The apparent unfolding thermodynamic parameters (Tables 2 and 4) and the unfolding and conformational transition pathways (Figure 9) clearly indicate that the interactions between the linker region and the Palm domain were disrupted prior to the unfolding of the four domains of Dpo4. The apparent T_m values (Table 2) and the FTS analysis (Figure 6) demonstrate that the LF domain is the most stable domain during the thermal denaturation process. Although the thermal unfolding order of the three domains in the Core fragment was not elucidated in this paper, wt Dpo4 was irreversibly heat-denatured by the following sequence: the linker region, then the Core, and finally the LF domain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CD	circular dichroism
Core	the Thumb, Finger, and Palm domains of Dpo4
Dpo4	<i>Sulfolobus solfataricus</i> DNA Polymerase IV
FTS	fluorescence-based thermal scanning
LF	the little finger domain of Dpo4
LF⁺	the linker region and the little finger domain of Dpo4 with
wt	wild type

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Figure 1.

Conformations of the LF domain relative to the Core of Dpo4. The crystal structures of the (a) apo-state (PDB ID: 2RDI),²⁸ (b) PCNA-bound state (PDB ID: 3FDS),²⁹ and (c) binary complex with DNA (PDB ID: 2RDJ)²⁸ are shown. The Finger, Palm, Thumb, and LF domains are colored in blue, red, green and purple, respectively. The linker region is colored black, the two subunits of heterotrimeric *S. solfataricus* PCNA is shown in yellow, and the DNA substrate is in grey.

Figure 2.

Diagram of wt Dpo4 and its truncation mutants. The residue numbers, the names of structure domains, and the names of fragments are denoted. The Finger, Palm, Thumb, and LF domains are colored in blue, red, green and purple, respectively, while the linker region is represented as a black line.

Figure 3. CD spectra of wt Dpo4 and its mutants at 37 °C. Far-UV regions of the CD spectra of wt Dpo4 (solid black line), the Core (solid blue line), the LF⁺ (dashed purple line), the LF (dotted red line), and the All-Gly linker mutant (dashed light blue line) are shown.

Figure 4. Thermal denaturation of wt Dpo4 and its truncation mutants monitored via CD spectroscopy at a fixed wavelength of 222 nm. The plots of wt Dpo4 (black ●), the Core (blue ■), the LF (red ◆), and the LF⁺ (purple ▲) are shown.

Figure 5.

Calculations of thermodynamic parameters for thermal denaturation of wt Dpo4. (a) Van't Hoff plots ($\ln(K_T)$ versus $1/T$) and (b) Gibbs free energy plots (ΔG_m versus temperature) of the first transition (■, black color), the second transition (◆, blue color), and overall unfolding (●, red color) for wt Dpo4. ΔS_m , ΔH_m , and T_m were calculated based on the linear fit of the plots.

Figure 6. Thermal denaturation of wt Dpo4 and its truncation mutants monitored via FTS. The FTS traces for wt Dpo4 (black ●), the LF⁺ (blue ■), and the LF (green ◆) are shown.

Figure 7.

Comparison of the thermal denaturation plots of wt Dpo4 and its linker region mutants monitored via CD spectroscopy at a fixed wavelength of 222 nm. (a) Thermal denaturation plots of wt Dpo4 (●, same as Figure 4) and its mutants: the All-Gly linker (light blue ■), the P236A (red ◆), the R/K-to-A linker (dark blue ■), and the R/K-to-D linker (purple ▲). (b) Thermal denaturation plots of wt Dpo4 (●, same as Figure 4) and its mutants: the R240A (purple ■), the E235A/R240A (light blue ◆), the E100A (green ■), K148A (red ▲), the E100A/K148A (orange ◆), the E100A/E235A/R240A (grey ▲), the K148A/E235A/R240A (blue ●), and the E100A/K148A/E235A/R240A (yellow ■).

Figure 8.

Interaction networks of the amino acid residues in the linker region and the Palm domain of Dpo4. (a) apo-state (PDB ID: 2RDI), (b) PCNA-bound state (PDB ID: 3FDS), and (c) DNA-bound binary complex (PDB ID: 2RDJ). The Palm and linker residues are shown in green with green labels and in grey with black labels, respectively (N atoms in blue and O atoms in red). Hydrogen bonding and ion pair interactions are shown as dotted lines. Residues are not considered to interact if their atoms are separated by more than 5 Å (see Supplementary Table 1).

Figure 9.

Unfolding and conformational transition pathways. (a) The irreversible heat-denaturation pathway is shown with the native (N), unfolding intermediate (I), and unfolded state (U). (b) The reversible conformational change pathway from the Apo to PCNA-bound state, and then to DNA-bound state is shown with two intermediate states (I') which are structurally similar to the unfolding intermediate (I) in (a). The Palm, Thumb, Finger and LF of Dpo4 are shown in red, green, blue, and purple, respectively. The linker region is colored in black, heterotrimeric PCNA is shown in yellow, and the DNA substrate is in grey. Interactions between residues in the linker region and Palm domain are represented as brown lines.

Table 1

Amino acid sequence of the linker region for specific Dpo4 mutants.

Enzyme	Linker Region Sequence ^a
wt Dpo4	RDEYNEPIRTRVRK
E100A	RDEYNEPIRTRVRK
K148A	RDEYNEPIRTRVRK
E100A/K148A	RDEYNEPIRTRVRK
E100A/E235A/R240A	RDEYNAPIRTAVRK
K148A/E235A/R240A	RDEYNAPIRTAVRK
E100A/K148A/E235A/R240A	RDEYNAPIRTAVRK
P236A	RDEYNEAIRTRVRK
R240A	RDEYNEPIRTAVRK
E235A/R240A	RDEYNAPIRTAVRK
R/K-to-A linker	A DEYNEPIATAV A
R/K-to-D linker	D DEYNEPI DTDVDD
All-Gly Linker	GGGGGGGGGGGGGGG

^aSite-specific mutations are in bold.

Table 2

Apparent unfolding thermodynamic parameters derived from thermal denaturation of each protein monitored by CD spectroscopy at 222 nm.

Protein		T_m^a (°C)	ΔH_m^b (kcal*mol ⁻¹)	ΔS_m^b (kcal*mol ⁻¹ *K ⁻¹)
wt Dpo4	Overall ^c	96 ± 1	67 ± 7	0.18 ± 0.02
	Native State	89.3 ± 0.2	516 ± 66	1.4 ± 0.2
	Intermediate	102.6 ± 0.1	162 ± 6	0.43 ± 0.02
Core		101.1 ± 0.1	94 ± 2	0.25 ± 0.01
LF		>105	-	-
LF ⁺	Overall ^c	98.1 ± 1.1	44 ± 7	0.12 ± 0.02
	Native State	86.8 ± 0.2	449 ± 55	1.2 ± 0.2
	Intermediate	105.6 ± 0.1	115 ± 3	0.30 ± 0.01
All-Gly linker		102.5 ± 0.1	73 ± 2	0.19 ± 0.01
P236A	Overall ^c	97.6 ± 0.9	50 ± 6	0.13 ± 0.02
	Native State	83.0 ± 0.2	130 ± 9	0.36 ± 0.03
	Intermediate	103.7 ± 0.1	190 ± 9	0.50 ± 0.02

^a Calculated at $\Delta G_m = 0$. Error derived from linear data fit.

^b Calculated using $\ln K_m = -\Delta H_m/(RT) + \Delta S_m/R$. Error derived from linear data fit.

^c Overall refers to calculations including the total temperature range of thermal denaturation. As no intermediate was observed for the Core, LF, and All-Gly linker mutant, only the overall T_m is given.

Table 3

Apparent unfolding thermodynamic parameters in the presence of 50 mM guanidine hydrochloride at 222 nm.

Protein	T_m^a (°C)	ΔH_m^b (kcal*mol ⁻¹)	ΔS_m^b (kcal*mol ⁻¹ *K ⁻¹)
wt Dpo4	99.6 ± 0.1	152 ± 4	0.41 ± 0.01
P236A	97.4 ± 0.1	174 ± 5	0.47 ± 0.01

^a Calculated at $\Delta G_m = 0$. Error derived from linear data fit.

^b Calculated using $\ln K_m = -\Delta H_m/(RT) + \Delta S_m/R$. Error derived from linear data fit.

Table 4

Apparent unfolding thermodynamic parameters derived from thermal denaturation of each protein monitored by CD spectroscopy at 222 nm.

Protein	T_m^a (°C)	ΔH_m^b (kcal*mol ⁻¹)	ΔS_m^b (kcal*mol ⁻¹ *K ⁻¹)
R/K-to-A linker	96.4 ± 0.2	89 ± 4	0.24 ± 0.01
R/K-to-D linker	104.4 ± 0.4	118 ± 8	0.31 ± 0.02
R240A	96.5 ± 0.3	93 ± 9	0.25 ± 0.03
E235A/R240A	90.3 ± 0.3	125 ± 6	0.34 ± 0.02
E100A	88.9 ± 0.2	163 ± 5	0.45 ± 0.01
K148A	92.8 ± 0.3	98 ± 6	0.27 ± 0.02
E100A/K148A	89.4 ± 0.2	135 ± 7	0.37 ± 0.02
E100A/E235A/R240A	88.3 ± 0.1	193 ± 5	0.53 ± 0.01
K148A/E235A/R240A	90.3 ± 0.3	172 ± 11	0.47 ± 0.03
E100A/K148A/E235A/R240A	90.0 ± 0.2	163 ± 5	0.45 ± 0.01

^aCalculated assuming $\Delta G_m = 0$. Error derived from linear data fit.

^bCalculated using $\ln K_m = -\Delta H_m/(RT) + \Delta S_m/R$. Error derived from linear data fit.