Modulation of the Structure, Catalytic Activity, and Fidelity of African Swine Fever Virus DNA Polymerase X by a Reversible Disulfide Switch*\$ [1]

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African swine fever virus polymerase X (pol X) is the smallest DNA polymerase known (174 amino acids), and its tertiary structure resembles the C-terminal half of prototypical X-family DNA polymerase known (174 amino acids), and its tertiary structure and its related conformational changes.

The African swine fever virus (ASFV)2 was first observed in Kenya in 1921 (1) and was described to cause infections of domestic pigs leading to a hemorrhagic fever, usually resulting in death. It is an economically significant disease with a potential of epidemic proportions resulting in losses of livestock. ASFV is a large icosahedral, cytoplasmic deoxyxivirus prone to interactions with host macrophages (2). Although genetically similar to the poxviruses, it is structurally closer to the iridoviruses but sufficiently different from both such that it has recently been classified as the founding member of its own family, the Asfarviridae family (African swine fever and related viruses) (3, 4).

The ASFV genome is a linear double-stranded DNA molecule of 170–190 kb (5), depending on the strain, coding for >150 proteins (6). About one-third of these proteins are required for the structural integrity of the virus (7, 8), with a number of enzymes involved in gene transcription or repair, protein modification, or interactions with the host (6). Replication of the ASFV virus occurs in macrophages (9, 10), which the virus enters through an unknown receptor, mediating endocytosis (11, 12). Early stage viral DNA replication is believed to occur in the nucleus (13, 14) followed by a later phase of replication in the cytoplasm, in what has been termed the cytoplasmic virus “factory” (15) or perinuclear inclusion bodies. These factories contain high levels of viral structural proteins, viral DNA, and membranous material needed for particle assembly (14, 16–20). A reducing environment in the virus factory is critical for the assembly in these early stages. However, the mature virion was shown to be resistant to oxidation upon release in the oxygen–rich cytosol or extracellular space (21). Maturation of the virus involves the initial formation of an empty particle within the virus factory, which is then filled by the separately formed nucleoproteins and viral DNA (22–24). Upon leaving the virus factory, these mature virions are infectious (25) and either remain within the cell or are transported to the plasma membrane, where they are released by budding (26) and act as infectious extracellular envelope virosomes.

The ASFV genome codes for a number of proteins vitally involved in DNA replication, including a minimal repair system (6), consistent with the later stage replication inside the perinuclear space. Pol X, one of the two DNA polymerases encoded in the ASFV genome, is a close structural analogue of the well known mammalian DNA polymerase pol β (27), involved in the BER pathway (28). Additional enzymes consistent with the presence of a viral BER pathway were found in the ASFV genome, i.e. an apurinic endonuclease, phosphodiesterase, and
were concentrated in Ultra-15 Amicon centrifugal filter units (10,000 NMWL, Millipore, Billerica, MA) in a swinging bucket rotor at 4,000 x g. Final volumes from 100 to 500 µl were used. Buffer exchanges were achieved by diluting the concentrated sample with 5 ml of the new buffer followed by a re-concentration step, repeating the procedure three to five times. All NMR samples contained 5% D₂O (v/v) as a lock substance.

NMR Spectroscopy—The oxidation state of pol X was monitored by ¹H-¹⁵N HSQC (39) experiments at 20 °C using an AVANCE spectrometer (Bruker BioSpin, Rheinstetten, Germany) at either 500.13 or 800.13 MHz. Both instruments were equipped with a triple resonance inverse cryogenically cooled NMR probe. The small samples used for biological assays were measured in 1.7-mm NMR tubes using the MATCH™ system (both Bruker Biospin, Billerica, MA), requiring only 40 µl of protein solution (at a concentration of 200 µM) in NMR buffer. All other measurements were done with samples containing about 500 µM protein solution in a volume of 300 µl of NMR buffer, prepared in 4-mm NMR tubes, inserted in a 5-mm outer tube (both Wilmad-LabGlass, Buena, NJ) that contained D₂O as lock solvent (40). Standard ¹H-¹⁵N HSQC measurements were applied utilizing a flip-back pulse and Watergate sequence for water suppression (39, 41–43). Further parameters were as follows: spectral width 12 ppm, 1024 complex points in the ¹H dimension, 32 ppm with 128 complex points in the ¹⁵N dimension, and a relaxation delay of 1.2 s. Data were processed and analyzed using NMRPipe and NMRDraw software (44). Data processing included zero filling in the acquisition dimension and linear prediction followed by zero filling in the indirect dimension, resulting in a data matrix of 2048 x 512, a π/2-shifted squared sine window function in both dimensions, and a time domain convolution function to suppress the residual water signal (45).

NMR experiments for chemical shift assignments of the backbone of an oxidized sample of pol X were performed at 20 °C on an AVANCE spectrometer (Bruker BioSpin, Rheinstetten, Germany) at 600.13 MHz, equipped with triple resonance inverse cryogenically cooled NMR probe. Resonance assignments for ¹H, ¹⁵N, ¹³C-α, ¹³C-β, and ¹³C-δ were made using the connectivities derived from the HNCACB, CBCAcoNH, HNCA, HNcoCA, HNcaCO, and HNCOCO spectra (46–53). The data were processed with Toppspin 2.1 (Bruker Biospin, Billerica, MA) and analyzed with NMRView 8.0.a18 with Java 1.6.0.01 (OneMoon Scientific, Newark, NJ) (54). Figures of molecular structures were prepared using PyMol (The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA).

EXPERIMENTAL PROCEDURES

Enzymes—Heterologous expression of isotopically labeled ¹⁵N and ¹³C/¹⁵N/¹³C full-length ASFV pol X (residues 1–174) was done in Escherichia coli as described (34, 36–38). Enzyme purification was as described (36, 38), but 20 mM PIPES-Na buffer was used throughout the purification instead of phosphate. The standard NMR buffer contained 20 mM PIPES-Na (pH 6.5), 20 mM MgCl₂, 0.02% NaN₃ (w/v), 0.5 mM NaCl, and 5 mM DTT. DTT (up to 100 mM) was added to the buffer to ensure fully reduced samples, whereas buffer without DTT was used for oxidized pol X samples. To expedite the oxidation, the buffer was oxygen-saturated by bubbling pure O₂ through the solution. Samples

were concentrated in Ultra-15 Amicon centrifugal filter units (10,000 NMWL, Millipore, Billerica, MA) in a swinging bucket rotor at 4,000 x g. Final volumes from 100 to 500 µl were used. Buffer exchanges were achieved by diluting the concentrated sample with 5 ml of the new buffer followed by a re-concentration step, repeating the procedure three to five times. All NMR samples contained 5% D₂O (v/v) as a lock substance.

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A 5’-GATCACAGTGAGTAC pACGACGGCCACT-3’
3’-CTAGTGTCACTCATG G TGCTGCGGTTGA-5’

B 5’-GGGGGAAAGGATTC -3’
3’-CCCGCCAATCAGGTTAC-5’

FIGURE 1. Schematic representation of gapped (A) and nongapped (B) DNA substrate. The 15- and 13-mer primers, respectively, were 32P-labeled, and an unlabeled 5’-phosphorylated downstream oligonucleotide was used, forming a single base gap opposite G16 in the template.

Steady-state Kinetics—pol X-catalyzed single nucleotide incorporation was measured over a range of dNTP concentrations for both primer-template (13/18-mer) and gapped (15/12-mer) DNA substrates. Two samples of purified pol X were prepared, one under reducing conditions (100 mM DTT) and one in which the enzyme was in the completely oxidized state, both verified by NMR. All reactions for the reduced enzyme were carried out at 37 °C in 50 mM Tris-HCl buffer (pH 7.4) containing 100 μM Mg2+/H11002, 10 mM dNTP (v/v), 50 mM NaCl, 100 μM iodoacetamide. LC-MS/MS analysis of the peptides was performed using an LTQ-Orbitrap mass spectrometer (Thermo-Fisher, Waltham, MA) equipped with an Eksigent NanoLC-As1 Autosampler 2.08, the Eksigent NanoLC-1D plus high performance liquid chromatography pump (Eksigent, Dublin, CA), and Nanospray source. The peptides were resolved on a packed fused silica capillary column, 100 μm × 4 cm, packed with octadecylsilane (C18) resin (Jupiter C18, 5 μm, 300 Å, Phenomenex, Torrance, CA), and coupled with an inline trapping column (100 μm × 4 cm) packed with the same C18 resin (using a frit generated with liquid silicate Kasil (55)). LC was carried out at ambient temperature with a flow rate of 0.5 μl/min using a gradient mixture of 0.1% (v/v) HCO2H in H2O (solvent A) and 0.1% (v/v) HCO2H in CH3CN (solvent B). The flow rate during the loading and desalting phase of the gradient was 1.5 μl/min and during separation phase was 500 nl/min. A 95-min gradient was performed with a 10-min washing period diverted to waste after the pre-column (100% solvent A for the first 10 min followed by a gradient to 98% solvent A, v/v, at 15 min) to allow for removal of any residual salts. After the initial washing period, a 60-min gradient was performed where the first 35 min was a slow, linear gradient from 98% solvent A to 75% A, followed by a faster gradient to 10% solvent A at 65 min and an isocratic phase at 10% solvent A to 75 min. Orbitrap full MS scans were collected in profile mode at 60,000 resolution and a maximum injection time of 1 s. Data-dependent MS/MS scans were acquired for the five most intense ions from the full scan using an isolation width of 2 m/z, an activation time of 30 ms, a 30% normalized collision energy, and a maximum injection time of 150 ms. Charge states of 1+ and >+4 were excluded from consideration for MS/MS, and a dynamic exclusion list of 50 ions was used to allow collection of MS/MS spectra from lower abundance peptides. The instrument was operated in preview mode to allow for concurrent collection of MS/MS spectra during the Orbitrap scan.

RESULTS

Oxidized and Reduced Forms of ASFV pol X—A comparison of various 1H-15N HSQC data collected on pol X indicated that a number of amide resonances were shifting with time (Fig. 2). The chemical shifts from a freshly prepared pol X sample were
in good agreement with the data deposited at the Biological Magnetic Resonance Bank by Maciejewski et al. (36, 37), but this was no longer the case after 4–6 months for those shifting peaks. Between measurements, the sample was maintained without further manipulation at 4 °C in the standard buffer, containing 5 mM DTT. Although DTT is a useful reducing agent, oxidation has been reported in aerated solutions over time (56–58), depleting the reducing potential in the solution and allowing cysteines to be oxidized. Furthermore, the reducing power is decreased at pH values <7, where the reactive thiolate form is protonated. With no other changes apparent (except for a potential exposure of the sample to air), the hypothesis that the observed chemical shift perturbation was caused by a slow oxidation of cysteines present in pol X seemed reasonable. The structure of pol X, as reported by Maciejewski et al. (36, 37), revealed that the only two cysteines present in pol X are located in the β-sheet of the palm domain. The two sulfur atoms are separated by about 7 Å, sufficiently close to form a disulfide bond. Further support for the hypothesis comes from a structure of pol X published by Showalter et al. (30). The most significant difference between the two structures was an observation of a disulfide bond in the structure reported by Showalter et al. (30). The main evidence for including a disulfide bond was based on two downfield-shifted 13C-β chemical shifts for both cysteines. Interestingly, no further elaboration of the fact that pol X indeed may exist in a reduced or oxidized form has appeared in the succeeding literature.

The 1H-15N HSQC spectra acquired at different times (Fig. 3) demonstrate the substantial chemical changes for two distinct correlations, amides Val-65 and Lys-85. Their correlation peaks moved from the initial positions (Val-65, 118.4, 6.89 ppm; Lys-85, 119.0, 6.93 ppm) to new positions (Val-65, 118.0, 6.83 ppm; Lys-85, 119.8, 7.07 ppm). The series of spectra (Fig. 3, A–D) was recorded on a doubly labeled (13C, 15N)-labeled pol X sample over a period of 5 months. All spectra were acquired with both 13C and 15N decoupling, and the sample was stored at 4 °C between measurements. The main peaks (Fig. 3A) were assigned to the reduced form according to Maciejewski et al. (36, 37), although the small peaks in that spectrum were anticipated to belong to the oxidized species. The relative intensities of the peaks were comparable for the freshly prepared sample and after 1 month of storage (Fig. 3, A and B, respectively), indicating that 5 mM DTT was sufficient to retain the majority of the sample in the reduced state during this time. A major shift in peak intensity occurred between the 1st and 4th months (Fig. 3, B and C, respectively). The initially strong peaks were reduced significantly, and the small peaks of the putative oxidized species became the main peaks. Two weeks later (Fig. 3D), signals of the initially dominant peaks were no longer observed. This conversion indicated that the DTT was oxidized and no longer acting as a reducing agent, and hence oxidation of pol X occurred. Spectra recorded after 8 and 12 months revealed no further change.

The observation of potential oxidation of the cysteines (i.e., Cys-81 and Cys-86) over time is unprecedented for DNA polymerases. Because of its biological implications, careful investigations were performed to unambiguously determine the presence of these two forms, using three independent methods as follows: 1) 1H-15N HSQC monitoring; 2) chemical shift analysis of amide and cysteine resonances; and 3) measuring the mass difference by MS after iodoacetamide alkylation followed by protein digestion.

1H-15N HSQC Monitoring—A freshly prepared sample of 15N-labeled pol X in standard buffer conditions was used. To obtain the fully oxidized form of pol X within days instead of months, DTT-free NMR buffer was saturated with oxygen. This exchange was accomplished by concentrating the sample in an Amicon Ultra-15 centrifugal device five times (to 0.3 ml) and subsequently diluting it each time (to 5 ml) with the new buffer. The 1H-15N HSQC NMR spectrum (measured on a DRX 500 instrument equipped with a cryoprobe) on this freshly prepared sample showed the expected peaks corresponding to the reduced form (supplemental Fig. S1A) and a second set of weaker peaks corresponding to the already present oxidized form (also see Fig. 3). Subsequent spectra were acquired, and the chemical shifts of the correlation peaks Val-65 and Lys-85 were monitored. In this oxygen-enriched environment devoid of any reducing agent, a shift in peak intensity analogous to the one described above was observed within days (supplemental Fig. S1A–E), compared with weeks in the preliminary trials.

Reversibility of this process was demonstrated on a 34-μl aliquot of the above NMR sample after the addition of 2 μl of
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D₂O and 4 μl of 1 mM DTT. The peaks completely reverted to the original positions within 3 days (supplemental Fig. S1F–H). This observation was not limited to the Val-65 and Lys-85 peaks but occurred throughout the spectrum and showed that changing only the oxidation potential of the buffer moved the equilibrium of pol X between two sets of peaks. Because the addition of DTT was the only change leading to this transition, the two forms must represent the oxidized and reduced forms. The most likely place for such a redox reaction is between the (only) two cysteines, which was proven by MS analysis (see below). Interestingly, even in the presence of O₂-saturated buffer and no reducing agent (or a large excess of reducing agent, e.g. 100 mM DTT), the conversion takes days. Such a slow conversion indicates that the disulfide bond formation is hindered by competing processes, e.g. conformational changes to accommodate the formation of the bond. The chemical shift is perturbed for a large number of amide peaks, further indicating a local rearrangement to accommodate either form.

This unique behavior of pol X demanded a careful validation of the oxidation state for each sample used. The ability to monitor the oxidation state by NMR within 1 h even for very small sample volumes (e.g. 40 μl of 0.2 mM pol X in a 1.7-mm NMR tube) proved to be very useful when dealing with the small samples required for MS analysis or biochemical activity and kinetic studies.

Chemical Shift Analysis of Amide and Cysteine Resonances in the Reduced and Oxidized Forms of pol X—The observation of chemical shift changes for a large number of amide resonances between the reduced and oxidized forms indicated the likelihood of a conformational change in β-sheet 8–10 (supplemental Fig. S2), where the two cysteines reside. Whereas the chemical shift data for the reduced form were available from the Biological Magnetic Resonance Bank (accession number 5010), no data were available for the oxidized form. Therefore, a full backbone assignment was done after careful verification that the freshly prepared ¹⁵N- and ¹³C-labeled sample was in the oxidized form. Standard backbone experiments (HNCACB, CBCACoNH, HNCoCACB, HNCA, HNCoCA, HNcACO, and HNCO) were performed at 20 °C. The data were processed with Topspin 2.1 and analyzed with NMRViewJ. With the exception of the prolines, Met-1, and Ser-39, all amide nitrogen and proton resonances were assigned. The backbone assignment was >99% complete, and a total of only seven shifts were missing. When including the C-β resonances, the completeness was >98%, with only 16 shifts missing (supplemental Table S1).

Chemical shift perturbation studies (59–64) between the oxidized and reduced form were carried out using the amide resonances, as determined for the oxidized form and utilizing the reported shifts for pol Xred (36, 37). Overall, the amide proton shift differences largely follow the ones observed for the ¹⁵N shifts, and a weighted and averaged chemical shift difference of the amide residues was used for the comparison (59–61). Good chemical shift agreement was seen for residues Leu-2/Asn-48, Leu-105/Leu-174, and Asp-49/Leu-74, with the exception of residues Val-50, Glu-58, Leu-62/Leu-66, and Arg-70/Ile-71 in this region, where differences >0.09 ppm occurred. The region between Val-78/Ala-104 contained fragments of residues with large chemical shift differences (>0.2 ppm), interrupted by those with minor changes, mainly between Ile-90 and Leu-99 (supplemental Fig. S3). Visualization of the chemical shift differences is presented as a ribbon diagram in Fig. 4, with Cys-81 and Cys-86 indicated by a stick representation, including the side chain atoms. Mean structures of pol X were calculated from the oxidized (Tsai and co-workers (30), PDB code 1JQR) and reduced forms (Mullen and co-workers (36), PDB code 1JAI). Where no amide shifts were available (either because of missing data for the reduced form by Maciejewski et al. (37), the prolines, and a missing Ser-39 in both forms), the chemical shift perturbation was introduced as a linear fit between the neighboring residues. Mapping of the chemical shift changes revealed that the largest changes occurred in the vicinity of the two cysteines and were propagated to the adjacent strands of the β-sheet containing strands 6, 8, 9, and 10 (Fig. 4B).
TABLE 1
Chemical shift data indicating the oxidation state(s) of Cys-81 and Cys-86

|       | C-α      | C-β       |
|-------|----------|-----------|
| ppm   | ppm      |           |
| Sharam et al. (65) |          |           |
| S–H (β) | 56.4 ± 1.8 | 29.7 ± 2.0 |
| S–S (β) | 54.9 ± 2.1 | 43.0 ± 4.2 |
| Reduced (Maciejewski et al. (36, 37) and this work) |  |  |
| Cys-81 | – | 59.2† |
| Cys-86 | – | 56.1† |
| Oxidized (Showalter et al. (30) and this work) |  |  |
| Cys-81 | – | 58.2† |
| Cys-86 | 57.0† | 47.6 | 47.2† |

† Data are from this work.

would be expected to be the case if a rearrangement of this sheet is required to accommodate formation of the disulfide bond. Indeed, the large perturbations around the cysteines observed upon addition of oxidizing or reducing agents further confirms that a change in the oxidation state(s) of Cys-81 and Cys-86 is the likely cause of a conformational change in the β-sheet of the palm domain of pol X. Also, the loop between the two cysteines experiences a major shift projecting into the subdomain interface in the reduced structure (Fig. 4C) while being flipped back toward the α-helix C in the oxidized form (Fig. 4B).

A comparison of the carbon chemical shifts for both forms provides another indication for the presence of two oxidation states in pol X. Examination of the 13C-α and 13C-β shifts reported in the literature and measured here (Table 1) indicates that although the 13C-α shifts are less characteristic of disulfide formation, an extreme downfield shift in the 13C-β shift value identifies the disulfide bond as such (65, 66). This is the case for the pol Xox sample with measured 13C-β shifts of 47.2 and 50.1 ppm for Cys-81 and Cys-86, respectively, leading to the conclusion that this form contains a disulfide bond. The shifts also match the ones reported by Showalter et al. (30) (Table 1), which was the basis for introduction of a disulfide bond into the structure calculation in that report. Chemical shift data for the reduced Cys-81 were not reported by Maciejewski et al. (37), but 13C-β shifts obtained in the present work of 28.7 and 31.0 ppm for Cys-81 and Cys-86, respectively, support the reduced form well (Table 1).

Oxidation and Reduction of pol X Complexed to Nucleoside Triphosphates—The β-sheet in the palm domain of pol X contains three aspartic acids (Asp-49, Asp-51, and Asp-100), whose homologues in pol β have been shown to play important roles in the catalysis involving the incoming dNTP and are presumed to have a similar function in pol X. Recent findings by Tsai and co-workers (67) also showed that pol X might deviate from the established polymerase reaction mechanism in forming a potentially productive binary dNTP complex in the absence of oligonucleotide. In either case, the dNTPs are putatively interacting with this β-sheet which, as discussed above, contains pronounced conformational changes between the oxidized and reduced form. Therefore, it was of interest to determine whether such a binary complex might impact the ability to form both oxidation states or vice versa. Both oxidation states can be readily achieved in the presence of dGTP when the sample is subjected to reducing or oxidizing conditions, as described for the apo-form (Fig. 5). Monitoring of the oxidation state could be done by observing Val-65 and Lys-85. Together with a number of other HSQC resonances, these two peaks experienced chemical shift perturbations, indicating a larger impact of the dNTP binding to the pol X–dNTP conformation. Likewise, the presence of both oxidation states in binary complexes with the other dNTPs and oligonucleotides was observed as well (results not presented).

MS Analysis—To determine the presence of the disulfide and to characterize differences between pol Xox/red forms, a shotgun LC-MS/MS experiment was performed on trypsin-generated peptides generated from these 15N-labeled proteins. The oxidation state of each sample was verified by NMR. The analyses were carried out using a Thermo LTQ-Orbitrap mass spectrometer, which provided not only a high resolution/high accuracy mass for a given peptide but also readily interpretable MS/MS spectra for the peptides present within the sample. The expected precursor mass of the disulfide cross-linked tryptic peptides was observed at <2 ppm mass difference of the predicted mass and was confirmed by the presence of the predicted disulfide cross-linked fragment ions in the corresponding MS/MS spectrum (Fig. 6 and high resolution MS in supplemental Fig. S4A). To demonstrate the presence of reduced cysteine residues in the pol Xox protein, the samples were alkylated prior to digestion. The presence of the carboxamidomethylated peptide was confirmed both by MS/MS and accurate mass of the parent peptide (supplemental Fig. S4, B and C). Taken together, the mass spectrometry data confirm the presence of both the disulfide cross-linked form in pol Xox and the free Cys-86 in the pol Xred sample.

Oxidized pol X More Actively Inserts and Extends Gapped DNA Substrates than the Reduced Enzyme—pol X-catalyzed insertion and extension (i.e. strand displacement) on gapped DNA substrates were allowed to proceed in the presence of all four dNTPs (Fig. 7), pol Xox inserted more readily and extended the gapped DNA when compared with the reduced form of the enzyme. Furthermore, pol Xox could perform some strand displacement activity (1–2 nucleotides), whereas the reduced form did not within the time course measured (1 h). Single nucleotide insertion of dCTP opposite dG was also more rapid.
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Kinetic Analysis of Oxidized and Reduced pol X Catalysis Reveals Differences in Fidelity—Steady-state kinetic analysis of single nucleotide incorporation reactions was used to quantify differences observed between the oxidized and reduced forms of pol X (Table 2). Both forms of pol X were more active on gapped DNA substrates that possess a 5′-phosphorylated oligonucleotide downstream of the one nucleotide gap, consistent with previous results (29, 31, 33, 34). With gapped DNA substrates, insertion of dCTP opposite G by pol Xox was only 13-fold more efficient than misinsertion of dGTP opposite G. The maximum rate, $k_{cat}$, was 4.6-fold greater for pol Xox, but the overall catalytic efficiency of the two enzyme forms was similar for inserting dCTP opposite G because of a reduced $K_{m,dNTP}$ for the reduced form (Table 2). With normal primer-template DNA, it was impossible to directly compare fidelity because pol $X_{rew}$ did not perform misinsertion events even after incubations as long as 8 h (results not shown). It should also be noted that previous studies have performed what were referred to as “pre-steady-state” time courses that extended to 5 min or more (29, 33). Such analyses are most likely not pre-steady-state conditions, because multiple binding/dissociation events presumably occur on this time scale. Fitting the results obtained for dCTP and dGTP insertion by pol Xox to a single exponential, we obtained “$k_p$” values (2.0 and 0.011 min$^{-1}$ for 1 mM dCTP and dGTP, respectively; supplemental Fig. S5) that were similar to the “$k_{pol}$” values previously reported (29). These data represent a composite of multiple kinetic equilibria (essentially measuring the specificity constant, $k_{cat}/K_m$) because the experimental conditions do not remove rate constants that define substrate dissociation events from consideration, i.e. these are not pre-steady-state measurements and should not be interpreted as such.

**DISCUSSION**

Mechanisms of genomic replication have been studied in great detail (68). Mutagenesis can result from a variety of causal events, e.g. covalent modification of DNA by exogenous/endogenous sources and failed or error-prone repair mechanisms. The intrinsic mechanistic features of different DNA polymerases can play a central role in many mutagenic events. Therefore, a detailed understanding of how these enzymes function during replication and repair is of great interest. The X-family DNA polymerases are a class of enzymes normally associated with DNA repair pathways such as BER (69, 70). ASFV pol X has served as an interesting mechanistic example of a polymerase because it only possesses two of the three ubiquitous polymerase domains and because it is unusually error-prone.

pol X lacks the thumb domain seen with most DNA polymerases and is, not surprisingly, a very distributive enzyme because the thumb domain normally makes substantial contact with the double-stranded portion of the DNA template. However, pol X does possess a finger domain (or dNTP selection domain), which has been shown to be important for determining the fidelity of other polymerases (71–73). What we find most unusual about pol X is the presence of a reducible disulfide bridge that occurs between Cys-81 and Cys-86 (results not shown). Redox control of catalytic activity has been observed previously with other nucleic acid-modifying enzymes, such as *E. coli* DinG helicase (74), but we are not aware of any precedent for disulfide-dependent modification of DNA polymerase activity. The two structures of pol X that have been published (30, 36, 37) point to the presence of oxidized and reduced forms of the polymerase. However, the only indication for their presence was given by a downfield-shifted $^{13}$C-β chemical shift of the two cysteines, and this observation had not been emphasized. We have unambiguously confirmed the presence of oxidized and reduced forms of pol X by NMR and MS methods and demonstrated that the oxidation/reduction occurs on Cys-81 and Cys-86. The distinction is important because we have shown that depending on experimental conditions, e.g. varying buffers or sample age,
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table 2

steady-state kinetic parameters for 1-base incorporation by pol x

| substrate  | dNTP       | k_cat  | k_m INSTP | k_cat/k_m INSTP | Δefficiency relative compared with dCTP:G |
|------------|------------|--------|-----------|-----------------|-------------------------------------------|
| oxidized   |            |        |           |                 |                                           |
| 15/12/28   | dCTP       | 2.3 ± 0.1 | 45 ± 9     | 0.051           | 13-Fold less                              |
| 15/12/28   | dGTP       | 0.036 ± 0.001 | 9.1 ± 0.8 | 0.0039          | 13-Fold less                              |
| 15/12/28   | dATP       | 0.0013 ± 0.0001 | 0.33 ± 0.17 | 0.0039          | 13-Fold less                              |
| 15/12/28   | dTTP       | 0.015 ± 0.001 | 430 ± 50   | 0.000035        | 1,500-Fold less                           |
| reduced    |            |        |           |                 |                                           |
| 15/12/28   | dCTP       | 0.51 ± 0.01 | 6.6 ± 1.3  | 0.077           | 107-Fold less                             |
| 15/12/28   | dGTP       | 0.0063 ± 0.0005 | 8.7 ± 4.7  | 0.00072         | 2,200-Fold less                           |
| 15/12/28   | dATP       | 0.0043 ± 0.00002 | 12.4 ± 6.7 | 0.000035        | 9,300-Fold less                           |
| 15/12/28   | dTTP       | 0.0015 ± 0.0005 | 120 ± 30   | 0.000083        |                                           |
| oxidized   | 13/18      | dCTP       | 0.31 ± 0.01 | 5.9 ± 0.8     | 0.052                                     |
| 13/18      | dGTP       | 0.0061 ± 0.00005 | 1.9 ± 0.9  | 0.00043         | 123-Fold less                             |
| 13/18      | dATP       | 0.0005 ± 0.0008 | 9.1 ± 2.7  | 0.00023         | 228-Fold less                             |
| 13/18      | dTTP       | 0.0002 ± 0.0003 | 19.7 ± 14.2 | 0.000012       | 4,500-Fold less                           |
| reduced    | 13/18      | dCTP       | 0.042 ± 0.003 | 11.7 ± 6.5   | 0.0036                                   |
| 13/18      | dGTP       | <0.0002    | <10⁻⁸      | >36,000-Fold    |
| 13/18      | dATP       | <0.0002    | <10⁻⁸      | >36,000-Fold    |
| 13/18      | dTTP       | <0.0002    | <10⁻⁸      | >36,000-Fold    |

FIGURE 8. Comparison with pol β ternary structure suggests a mechanism for altered fidelity in pol X. A, previously reported solution structures of pol X in both the reduced (magenta, PDB code 1JAJ) and oxidized (cyan, PDB code 1JQR) forms are shown in schematic form superimposed with pol β (PDB code 1BYP). The functional domains of pol β are highlighted for clarity. B, superimposing the palm domains of pol Xred (magenta) and pol Xox (cyan) with the ternary structure of pol β illustrates a potential interaction between the dNTP and the enzyme. Reduction of the disulfide bridge between β8 and β9 results in a shift toward the DNA template, which repositions the side chain of Lys-85 closer to the primer terminus and the incoming dCTP (green). The loss of the disulfide bond also shifts Asp-100 closer to the catalytic magnesium ion (yellow spheres).

either form might be present but not easily recognized. Even the presence of some of a reducing agent such as DTT will not prevent oxidation over time, which may be the reason why Showalter et al. (30) found the oxidized form in a low DTT-containing sample (1 mM), whereas Maciejewski et al. (36, 37) observed the reduced form (10 mM DTT). Furthermore, we observed that the addition of dNTPs in the buffer system does not block the conversion between reduced and oxidized pol X (Fig. 5). The putative binding site of the dNTPs is located at the β-sheet comprising the palm domain, which includes the catalytic triad (Asp-49, Asp-51, and Asp-100). Interestingly, Asp-100 is among the residues with the largest chemical shift differences between the oxidized and reduced form, being located directly across from Cys-88 and Val-89 on strand β10 (Fig. 4, B and C, supplemental Fig. S3 and S4).

Steady-state analysis of the two forms of pol X revealed that the reduced form was ~10-fold more accurate with gapped DNA substrates than the oxidized form (Table 2). The difference in fidelity observed for pol Xox and pol Xred may partially explain some of the discrepancies reported in the literature. The structural changes that occur in the palm domain provide a molecular basis for the distinct fidelities observed with the oxidized and reduced forms of pol X (29, 31). Superimposition of either pol X structure with the ternary structure of pol β shows the similar overall topology of the palm and finger domains (Fig. 8A). In focusing upon the region of the palm domain that undergoes the most dramatic conformational changes (namely β8, β9, and β10), at least two potentially important changes may be noted by adding the DNA substrate and incoming dCTP relative to the pol β ternary complex. The first observation relates to the obvious shifts of β8, β9, and (to a lesser extent) β10 and β6 toward the putative dNTP binding cleft of pol X as the enzyme goes from the oxidized to the reduced form (Fig. 8B), impacting the catalysis. The repositioning of β10 and β6 also results in a switch in the side chain orientation of the three aspartates (Asp-49, Asp-51, and Asp-100) that play a critical role in catalysis with pol β (28, 71) and could affect coordination of the catalytic metal ion (Fig. 8B) in pol X. The positions and distances between the carboxyl carbons of the three pol Xox side chains are in better agreement with pol β than pol Xred, which is furthest shifted in the direction of helix B, and the distance between these carbons for Asp-49 and Asp-51 is >2.5 Å larger than in pol β and pol Xox. Likewise, the pol β residues Ser-180 and Arg-183 were shown to play significant roles in catalysis by forming hydrogen bonds with the oxygens of the (dNTP) γ- and β-phosphates (75, 76). The conserved side chain analogues of pol X (Ser-39 and Arg-42) also show substantial displacement to either side of the pol β residues. These differ-
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ences are believed to modify catalysis and might impact the binding of the nucleotide, hence modulating catalytic efficiency and fidelity. The second observation deals with modified DNA-protein interactions. The reoriented β8 and β9 sheets place the side chains of Glu-83 and Lys-85 in a better position to form hydrogen bonds with the primer-template DNA. In addition to these changes, Lys-59, located in α-helix C and adjacent to β8 and β9, moves into a position that may be more favorable for interactions with the DNA substrate, consistent with previous results (77–79). In addition Lys-79, located in β8, undergoes substantial rearrangements when comparing the oxidized and reduced structures, although it is somewhat further removed from the dNTP binding cleft than Lys-85. Although all of these changes are observed in the apo-structures of pol X, it is reasonable to propose that they also occur in the pol X ternary complex, given the distinct kinetic profiles of the reduced and oxidized forms and because the overall topology of apo-pol X is similar to the ternary complex of pol β used in the superimposition.

It should be noted that there are clashes between the side chains of reduced pol X and the superimposed DNA, which would seem to indicate that the DNA substrate orientation for pol Xred is slightly different from what is observed in the pol β ternary structure, due to the dislocation of the β-sheet consisting of strands 6 and 8–10. Alternatively, the changes observed in the apo-form of reduced pol X may not be as pronounced when DNA is present. Nevertheless, it is possible that reorienting the DNA relative to Glu-83 and Lys-85 could place one or both of these two residues in closer proximity to the nascent base pair than observed in our superimposition. Such an occurrence would be consistent with roles for these residues in the increased fidelity that was observed for pol Xred. It is clear that going from the oxidized to the reduced state changes the conformations of Glu-83 and Lys-85 relative to the DNA substrate, but defining the precise protein/DNA contacts involved awaits further investigation. Based on our functional results it is reasonable to predict an important role for redox-induced conformational changes that reposition Glu-83, Lys-85, Asp-49, Asp-51, and Asp-100 (as well as Ser-39 and Arg-42) during catalysis and modulate the fidelity of pol X.

We have focused upon the palm domain of pol X because very few changes are observed in the finger domain when comparing the oxidized and reduced forms. Sequence and structural alignment of pol X and pol β previously suggested that the side chains of His-115 and Arg-127, located in the pol X finger domain, probably serve functions analogous to Tyr-271 and Arg-283 by forming contacts with the primer terminus and template base, respectively (33). However, the check upon the incoming dNTP provided by Asn-279 in pol β has no obvious analogue in the pol X structure, consistent with the reduced fidelity of the viral polymerase.

Other members of the DNA polymerase X family were examined for the presence of disulfide bonds. pol β contains three cysteines, which are spaced too far apart in the molecule to form a disulfide, and none have been reported in any of the numerous published crystal structures. Other DNA polymerases from the X-family include pol λ, pol μ, TdT, and the poorly characterized pols σ1 and σ2 (TRF4–1 and TRF4–2, respectively) (80, 81). pols λ and μ and TdT all have 7 cysteines, and pols σ1 and σ2 have 6 and 5 cysteines, respectively (UniProt accession Q5XG87 and Q8NDF8, respectively). In murine TdT, four of the seven cysteines are in the palm and thumb subdomains (PDB code 1JMS (82)). Interestingly, two of them, Cys-378 and Cys-404, are adjacent to each other with a distance of 6.4 Å between the sulfur atoms, sufficiently close to form a disulfide bond. A third cysteine, Cys-438, is located in a loop adjacent to the β-sheet containing Cys-378 and Cys-404, but with a slightly larger distance (13 Å). This β-sheet and the position of the cysteines correspond to β8 and β9 of pol X, but all of the cysteines are in the reduced form. Similarly, 4 of the 7 cysteines in the human pol λ structure (PDB code 1RZT (83)) are located in the palm-finger (C-terminal) domain. Three of these (Cys-412, Cys-415, and Cys-425) are also located in the same β-sheets, with sulfur-sulfur distances of ~10.4 Å and have been represented in the reduced form. It was reported (83) that Cys-543 of pol λ, which resides in the finger domain, has a tendency to form intermolecular disulfide bonds, and therefore several structures of pol λ have been reported containing a C543A mutation. Only three cysteines, Cys-353, Cys-392, and Cys-411, are present in the palm domain of the murine pol μ structure (PDB code 2IHM (84)). However, without any major conformational changes, the presence of a disulfide bond in pol μ similar to the one found in pol X is unlikely.

The biological consequences of a disulfide switch as a determinant in polymerase function remain unclear. A recent study (21) showed that the redox environment of the ASFV plays a critical role in its development. During the assembly process of ASFV in the perinuclear space, a reducing environment is essential; otherwise, the virus capsid folds only slowly and incompletely, forming insoluble aggregates. Once the virus is assembled and mature, it is resistant toward oxidation and can be released either into the cytosol of the macrophage (85) or the extracellular space. The host cell for ASFV has been described to be the swine macrophage, which is an oxidizing environment, producing and releasing reactive oxygen species (6, 31, 86). Therefore it is expected that pol X is exposed to both a reducing environment in the development stage and an oxidizing one in the mature virus, but it remains to be determined whether pol X is present in all viral development stages and which form prevails at any stage. Although our results provide strong evidence for a distinct functional difference between the two forms of pol X, we can only speculate if they present different functionalities in vivo. However, the concept that the fidelity of a polymerase is dictated by the oxidation state of two cysteine residues appears to be novel and may have important consequences for our understanding of mutagenesis.

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