Altered Order of Substrate Binding by DNA Polymerase X from African Swine Fever Virus†

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Received April 24, 2008; Revised Manuscript Received May 29, 2008

ABSTRACT: A sequential ordered substrate binding established previously for several DNA polymerases is generally extended to all DNA polymerases, and the characterization of novel polymerases is often based on the assumption that the enzymes should productively bind DNA substrate first, followed by template-directed dNTP binding. The comprehensive kinetic study of DNA polymerase X (Pol X) from African swine fever virus reported here is the first analysis of the substrate binding order performed for a low-fidelity DNA polymerase. A classical steady-state kinetic approach using substrate analogue inhibition assays demonstrates that Pol X does not follow the bi-bi ordered mechanism established for other DNA polymerases. Further, using isotope-trapping experiments and stopped-flow fluorescence assays, we show that Pol X can bind Mg$^{2+}$·dNTPs in a productive manner in the absence of DNA substrate. We also show that DNA binding to Pol X, although rapid, may not always be productive. Furthermore, we show that binding of Mg$^{2+}$·dNTP to Pol X facilitates subsequent formation of the catalytically competent Pol X·DNA·dNTP ternary complex, whereas DNA binding prior to dNTP binding brings the enzyme into a nonproductive conformation where subsequent nucleotide substrate binding is hindered. Together, our results suggest that Pol X prefers an ordered sequential mechanism with Mg$^{2+}$·dNTP as the first substrate.

DNA polymerases constitute an important class of enzymes and are required for genomic replication and maintenance. They catalyze template-directed nucleotidyl transfer reaction where a nucleotide (dNTP) is added to the 3′-OH terminus of a primer strand. Consistent with their importance to the viability of organisms, the function, structure, mechanism, and fidelity of DNA polymerases have been a focus of active research for several decades. In the early going, researchers established the order of DNA and dNTP substrate binding for several DNA polymerases. For example, steady-state kinetic studies showed that the substrate binding by Klenow fragment of Escherichia coli DNA polymerase I (PolI) is strictly ordered: the enzyme binds DNA first, followed by dNTP (I). Kinetic analyses of mammalian polymerases α and β and HIV reverse transcriptase also suggested the same sequential ordered mechanism in which DNA binding always occurs before productive dNTP binding (2–5). Structural studies have demonstrated that even though DNA polymerases can form binary complexes with dNTPs, the positioning of the substrate in the active site greatly differs from the catalytically potent conformation, which suggests nonproductive or adventitious substrate binding (6, 7). Furthermore, isotope-partitioning experiments using Pol I have demonstrated the catalytic incompetence of the E·dNTP binary complex, which in turn implies that any E·dNTP complex that may form must dissociate before DNA binding. It was also shown in the same studies that the enzyme should first form a productive binary complex with DNA substrate, followed by dNTP binding, for catalysis to occur (8).

On the basis of the aforementioned studies, it is generally assumed that all DNA polymerases possess the same sequential ordered mechanism, and the characterization of novel polymerases is often based on this premise (9–15). While this assumption is valid for DNA polymerases that exhibit high processivity, low to moderately processive DNA polymerases could, in principle, deviate from this strict “DNA-first” binding order. Recent discoveries of several DNA polymerases with novel properties such as low fidelity, a preference for certain dNTPs, selectivity for certain base pairs, and the use of damaged/unnatural substrates (14, 16–21) demand further investigations into their order of substrate binding.

DNA polymerase X (Pol X) from African swine fever virus (ASFV) is the smallest known DNA polymerase with 174 amino acid residues and a size of 20.3 kDa (22). Pol X is a very low fidelity polymerase and, most notably, catalyzes the incorporation of the dG:dGTP mismatch as efficiently as the dG:dCTP match (21, 23). Pol X also possesses moderate lesion bypass capabilities (18). On the basis of its
catalytic properties, Pol X has been implicated in mutagenic DNA repair and the viral hypermutagenicity (21, 23, 24). Pol X belongs to the X superfamily of nucleotidyltransferases (22, 25) and shares sequence and structural homology with mammalian Pol β but lacks the lyase domain and the N-terminal portion of the polymerase domain of the latter. Despite the lack of these structural elements, which are responsible for DNA binding in Pol β, Pol X is able to bind DNA with a very high affinity (26), largely due to its unique binding mode where it uses helix αE in the fingers subdomain, helix αC in the palm subdomain, and the interface of the palm and fingers subdomains to bind DNA (M.-I. Su et al., unpublished results; 26, 27). Pol X has also been shown to be a highly distributive enzyme (22), unlike Pol β, which has been shown to be processive on short gapped DNA substrates owing to the presence of its 8 kDa lyase domain. A recent study using a quantitative fluorescence titration technique showed that Pol X binds gapped DNA in two distinct modes: one utilizing the total DNA binding site of Pol X and the other utilizing only the auxiliary DNA binding site where the enzyme binds to the double-stranded region of the DNA (28).

NMR structural analyses of free Pol X have shown that it can bind dNTPs with high affinity in the presence of Mg²⁺ (27). Notably, binding of the purine nucleotides (Mg²⁺-dATP and Mg²⁺-dGTP) induces extensive ¹H and ¹⁵N chemical shift perturbations, propagating in both the finger and palm subdomains of Pol X (27). Further, modeling based on chemical shift perturbations suggested a conformational adjustment of subdomains upon Mg²⁺-dNTP binding; such rearrangements have not been observed for other polymerases in the absence of DNA (27). Even though these studies suggested that free Pol X can bind Mg²⁺-dNTP in its active site, it was not clear whether this binding is productive.

While all the DNA polymerases investigated to date seem to follow a sequential ordered mechanism, several related enzymes from the Pol X superfamily of nucleotidyltransferases have been shown to possess a sequential random mechanism (29–32). This suggests that the sequential ordered mechanism is not conserved throughout the nucleotidyltransferase superfamily. Further, this mechanism may not be required for nonprocessive DNA polymerases, lesion bypass DNA polymerases, and/or template-independent transferases.

In this study, we have used a classical steady-state kinetics approach to determine the kinetic mechanism of DNA and dNTP substrate binding to ASFV Pol X. Then we utilized substrate-trapping assays under pre-steady-state conditions to address the question of whether DNA or dNTP binding by Pol X results in a productive binary complex. Finally, using stopped-flow fluorescence technique, we analyzed the kinetics of DNA and dNTP binding by Pol X and the effect of substrate mixing order on the fluorescence transitions upon formation of the Pol X-DNA-dNTP ternary complex. Results of our kinetic analyses suggest that the order of substrate binding by ASFV Pol X differs from the bi-bi ordered mechanism suggested for other DNA polymerases.

MATERIALS AND METHODS

Materials. Ultrapure dNTPs and G-25 Microspin columns were purchased from GE Healthcare. [γ⁻³²P]ATP was purchased from MP Biomedicals. T4 polynucleotide kinase was obtained from New England BioLabs. BSA was from Roche. Reverse phase C18 cartridges were from Waters Corp. Materials and reagents not listed here were of standard molecular biology grade.

Purification of Enzymes. Recombinant ASFV Pol X was expressed and purified as previously described (21). The H115W mutant was generated by site-directed mutagenesis with forward primer 5’-GAAGAAAAACCATACGGCAATATTITGTTACGGTGCCCCTTC-3’ and reverse primer 5’-GAAACGGACCGTAAACCAAATATGGGTATGTTTTTTCCCTC-3’ using the QuikChange method (Stratagene). Successful mutagenesis was verified by sequencing of the plasmid. The mutant enzyme was expressed and purified as described for wild-type (WT) Pol X. The enzyme concentrations were determined by UV absorbance at 280 nm using extinction coefficients of 15930 and 21430 M⁻¹ cm⁻¹ for Pol X and the H115W mutant, respectively. The enzymes appeared to be homogeneous on the basis of SDS–PAGE developed using the silver staining method.

DNA Substrates. The sequences of DNA substrates used in this study are listed in Table 1. Custom synthesized oligomers were purchased from Integrated DNA Technologies (Coralville, IA). Each DNA oligomer was further purified with polyacrylamide–urea denaturing gels and extracted with 500 mM ammonium acetate and 1 mM EDTA. Extracted oligomers were subsequently desalted on a Sep-Pak C18 cartridge and eluted using a methanol/water mixture (60:40). After the solvent had been removed with a vacuum, the oligomers were resuspended in TE buffer [10 mM Tris-HCl and 0.1 mM EDTA (pH 8.0)], and the concentration was determined by UV using extinction coefficients provided by the vendor. The oligomers were then stored at −20 °C.

DNA substrates used in the chemical quench experiments were 5’-end-labeled using T4 polynucleotide kinase and [γ⁻³²P]ATP (4500 Ci/mol) according to the manufacturer’s protocol. The T4 polynucleotide kinase was inactivated by heating the solution at 65 °C for 20 min. The labeled primer was separated from unreacted ATP using a G-25 microspin column. Typically, only 5% of the substrate molecules were labeled with ³²P. All DNA substrates were assembled at room temperature by mixing primer, template, and downstream oligomers in a 1:1:1:1.2 molar ratio. Once assembled, substrates were stored at 4 °C prior to use.

Steady-State Inhibition Assays. Steady-state reactions were initiated by manually adding 0.3 nM Pol X to a reaction mixture containing 100 nM DNA substrate (or varied from 5 to 200 nM), 500 µM dNTP (or varied from 10 to 500 µM), and the appropriate concentration of inhibitor in a reaction buffer containing 140 mM KCl, 50 mM tris-borate, 3% glycerol, 10 mM MgCl₂, 100 µg/mL BSA, and 1 mM DTT (pH 7.5) at 37 °C. All concentrations listed above are postmixing. Aliquots (10 µL) of the reaction mixture were subsequently quenched by being mixed with 10 µL of formamide at time points ranging from 15 s to 60 min. Elongated DNA product in the quenched reactions was separated from the substrate DNA using 19% denaturing polyacrylamide gels. Formation of the pyrophosphate product was not monitored. Gel visualization was conducted by phosphor screen autoradiography using a STORM840 scanner from GE Healthcare. Band intensity quantitation and data plotting were performed using ImageQuant (GE Healthcare) and SigmaPlot 9.0 (Systat Software Inc.), respectively. DNA
product formation as a function of time was fit using linear regression to obtain the steady-state rate, $V_{\text{obs}}$. $V_{\text{obs}}$, as a function of varied substrate concentration, was then fit to the hyperbolic equation $V_{\text{obs}} = \frac{V_{\text{max}}[S]}{K_m + [S]}$, where [S] is the concentration of the varied substrate, to obtain steady-state kinetic parameters $V_{\text{max}}$ (maximum rate) and $K_m$ (Michaelis constant). $V_{\text{obs}}$ values obtained for each inhibitor—varied substrate pair were also fit globally to determine the type of inhibition and inhibition parameters using the following equations.

$$V_{\text{obs}} = \frac{V_{\text{max}}[S]}{K_m (1 + \frac{[I]}{K_i}) + [S]} \quad (\text{competitive inhibition})$$

$$V_{\text{obs}} = \frac{V_{\text{max}}[S]}{K_m + [S] (1 + \frac{[I]}{K_{II}})} \quad (\text{uncompetitive inhibition})$$

$$V_{\text{obs}} = \frac{V_{\text{max}}[S]}{K_m (1 + \frac{[I]}{K_i}) + [S] (1 + \frac{[I]}{K_{II}})} \quad (\text{noncompetitive inhibition})$$

where the inhibition constant, $K_i$, describes inhibitor binding to E alone and $K_{II}$ describes inhibitor binding to the E·S complex.

**DNA-Trapping Assay.** Solution A containing 400 nM Pol X and 200 nM 35(CG) DNA substrate [labeled with 32P (Table 1)] was rapidly mixed with solution B containing 1 mM ddGTP and 1 mM dGTP. In the control reaction, ddGTP was added to solution B and not to solution A. One nucleotide extension of the DNA substrate (incorporation of ddGTP and dGTP) as a function of time was fit to the double-exponential equation $[\text{DNA}_{n+1}] = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + C$, where $k_1$ and $k_2$ are rate constants of the first and second incorporations, respectively, and C is the concentration of the dd-terminated DNA product. 

**Stopped-Flow Fluorescence Assays.** Stopped-flow experiments were performed on an Applied Photophysics SX 18MV stopped-flow apparatus. For the 2-aminopurine (2-AP) fluorescence assays, the excitation wavelength was 312 nm with a spectral bandpass of 4 nm. 2-AP emission was monitored using a 360 nm high pass filter (Corion). For tryptophan (Trp) fluorescence experiments, the excitation wavelength was 290 nm with a spectral bandpass of 4 nm. Trp emission was monitored using a 335 nm high pass filter except that a 335–345 nm bandpass filter was used when 2-AP fluorophore was also present in the reaction mixture. All the reactions were performed in an assay buffer containing 140 mM KCl, 50 mM tris-borate, 3% glycerol, 10 mM MgCl$_2$, and 1 mM DTT (pH 7.5) at 37 °C. Typically, a minimum of seven runs were performed for each reaction and the traces averaged. The averaged traces were fit using...

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**Table 1: DNA Substrates**

| Designation$^a$ | Sequence$^b$ | Assay to be used in |
|-----------------|--------------|---------------------|
| 45(G)           | 5′-32P-GCTTGCGAGCTGCTCTCCAATCTCA GCTCGATCCATGCTCC | Substrate to be used in steady-state and DNA-trapping assay |
| 3′-CGGACGTCGGCTGGTGATGACCTTACGGCAAG | |
| 45(T)           | 5′-32P-GCTTGGCACGCTGCTCTCCAATCTCA GCTCGATCCATGCTCC | Substrate in steady-state assays |
| 3′-CGGACGTCGGCTGGTGATGACCTTACGGCAAG | |
| 35dd(G)         | 5′-GCTTGGCAGGCTGCTCTCCAATCTCA GCTCGATCCATGCTCC | Inhibitor in steady-state assays |
| 3′-CGGACGTCGGCTGGTGATGACCTTACGGCAAG | |
| 35(G)           | 5′-GCTTGGCAGGCTGCTCTCCAATCTCA GCTCGATCCATGCTCC | Non-labeled DNA in DNA-trapping assay |
| 3′-CGGACGTCGGCTGGTGATGACCTTACGGCAAG | |
| 35(CG)          | 5′-32P-GCTTGGCAGGCTGCTCTCCAATCTCA GCTCGATCCATGCTCC | Nucleotide-trapping assay |
| 3′-CGGACGTCGGCTGGTGATGACCTTACGGCAAG | |
| 36dd(T)         | 5′-GCTTGGCAGGCTGCTCTCCAATCTCA GCTCGATCCATGCTCC | 2-AP-dNTP fluorescence assays |
| 3′-CGGACGTCGGCTGGTGATGACCTTACGGCAAG | |
| 18dd/35AP       | 5′-GCTTGGCAGGCTGCTCTCCAATCTCA GCTCGATCCATGCTCC | 2-AP-DNA fluorescence assays |
| 3′-CGGACGTCGGCTGGTGATGACCTTACGGCAAG | |
| 35dd(GA)        | 5′-GCTTGGCAGGCTGCTCTCCAATCTCA GCTCGATCCATGCTCC | DNA binding fluorescence assays |
| 3′-CGGACGTCGGCTGGTGATGACCTTACGGCAAG | |

$^a$ Substrates with the dd designation have a dideoxy-terminated primer terminus. The number in designation is the total number of bases in the template. The templating base in the gap is shown in parentheses. $^b$ In the gapped DNA substrates, the downstream oligonucleotide is 5′-phosphorylated. A refers to a 2-aminopurine base. C indicates 2′,3′-dideoxy-2′-deoxycytidine 5′-monophosphate.
Inhibition Patterns Using a ddDNA Substrate Analogue with a Mismatch at the Templating Position. An essential feature of the ordered sequential mechanism is that the first substrate binding creates a binding site for the second substrate. Accordingly, the ddDNA substrate analogue with a mismatch at the templating position would have a lower affinity for the Pol X•dNTP binary complex and act as a poor inhibitor. We used 35dd(G) DNA as an inhibitor in Pol X-catalyzed incorporation of dATP into 45(T) DNA under steady-state conditions. As expected, the 35dd(G) DNA substrate analogue demonstrated competitive inhibition toward the 45(T) DNA substrate with a significantly higher $K_I$ (0.46 $\mu$M). The double-reciprocal plot when dATP was the varied substrate (Figure 1E) showed both the slope and the intercept effect. Consistently, the global analysis of the data suggested noncompetitive inhibition with $K_I$ and $K_{II}$ values of 0.29 and 0.84 $\mu$M, respectively, implying that the inhibitor binds to both the free Pol X and the Pol X•dATP complex. The fact that the same 35dd(G) DNA exhibited uncompetitive inhibition versus the varied dCTP substrate suggests that in the presence of matched dNTP the inhibitor binds primarily to the Pol X•dNTP complex utilizing a dNTP-directed DNA binding site. The noncompetitive pattern of inhibition in the absence of matched dNTP can be explained by the presence of an alternative Pol X—DNA binding mode which is independent of dNTP binding.

Inhibition by Pyrophosphate. Pyrophosphate (PP) is a product of the polymerase reaction and has been previously used as an inhibitor in mechanistic studies of DNA polymerases (1, 4, 29, 30). We attempted to use PP, inhibition in steady-state assays to gain further insights into the Pol X mechanism. However, we found that Pol X is not inhibited noticeably by up to 1 mM PP$_I$. At higher PP$_I$ concentrations, there was modest inhibition (data not shown); however, quantitative analysis of inhibition was not possible because of PP$_I$ solubility restrictions.

Inhibition Patterns Using a Mismatch Nucleotide. Previous kinetic studies have shown that Pol X displays a very high binding affinity for purine:purine mismatches under pre-steady-state conditions (21). Specifically, for a dG:dATP (template:incoming nucleotide) mismatch, Pol X displays tight binding ($K_{D,app} = 80 \mu$M) but a very slow turnover rate (430-fold slower than the rate of correct dG:dCTP incorporation). Accordingly, it is expected that dATP can act as an effective inhibitor in Pol X-catalyzed incorporation of dCTP into 45(G) DNA.

Using dATP as an inhibitor and dCTP as a varied substrate, the pattern of inhibition was competitive with a $K_I$ of 48 $\mu$M. There was no detectable incorporation under the steady-state conditions when dATP was used as the substrate in the absence of dCTP. These results confirmed that dATP can be used as a dCTP analogue in Pol X inhibition studies addressing the order of substrate binding.

The effect of incremental amounts of dATP on initial velocity, with DNA as the varied substrate, is shown in Figure 1F. If DNA were the leading substrate, dATP would exhibit uncompetitive inhibition toward DNA. However, the double-reciprocal plot exhibited the slope and intercept effect (Figure 1F), and the global regression fit best to a noncompetitive inhibition model with $K_I$ and $K_{II}$ values of 150 and
640 µM, respectively. This result provides strong evidence against the order of substrate binding proposed for other DNA polymerases, where DNA always binds before dNTP binding.

DNA-TRAPPING ASSAY UNDER PRE-STEADY-STATE CONDITIONS.
Substrate trapping is another common method used to understand enzyme mechanisms. DNA trapping has been used previously by researchers to establish the kinetic

FIGURE 1: Inhibition by ddDNA and mismatch dNTP in Pol X-catalyzed steady-state reactions. The concentrations used in the following reactions were 0.3 nM Pol X, 100 nM DNA substrate (or varied), and 500 µM dNTP substrate (or varied). All concentrations are after mixing. (A) Incorporation of dCTP into 45(G) DNA in the absence of inhibitor. dCTP concentrations were 10 (●), 20 (○), 50 (▼), 100 (△), 200 (□), and 500 µM (▲). The data were fit using linear regression to obtain \( V_{\text{obs}} \). (B) dCTP concentration dependence of \( V_{\text{obs}} \), obtained as described for panel A, in the presence of the following 35dd(G) inhibitor concentrations: 0 (●), 50 (○), 200 (▼), and 500 nM (△). \( V_{\text{obs}} \) values as a function of dCTP concentration were fit to a hyperbolic equation to provide \( k_{\text{max}} \) and \( K_m \) values listed in Table 2. (C) Double-reciprocal plots for assays with a constant dCTP concentration and varied 45(G) DNA substrate concentrations in the presence of 0 (●), 50 (○), 200 (▼), and 500 nM (△) 35dd(G) DNA inhibitor. (D) Double-reciprocal plots for assays with a constant 45(G) DNA concentration and varied dCTP substrate concentrations in the presence of 0 (●), 50 (○), 200 (▼), and 500 nM (△) 35dd(G) inhibitor. (E) Double-reciprocal plots for assays with a constant 45(T) DNA concentration and varied dATP substrate concentrations in the presence of 0 (●), 50 (○), 200 (▼), and 500 nM (△) 35dd(G) inhibitor with a noncomplementary templating base. (F) Double-reciprocal plots for assays with a constant dCTP concentration and varied 45(G) DNA substrate concentrations in the presence of 0 (●), 50 (○), 200 (▼), and 500 µM dATP (△).
mechanism for several DNA polymerases (8, 34). In these assays, the labeled DNA substrate is incubated with the polymerase and is then mixed with an excess of nonlabeled DNA and the correct nucleotide substrate. If the polymerase binds DNA first in a productive manner, there should not be a significant decrease in the burst amplitude of product formation when it is challenged with nonlabeled DNA. On the other hand, if DNA binding is nonproductive and DNA dissociation is required prior to formation of a productive complex, an excess of nonlabeled DNA should effectively compete for the enzyme active sites, resulting in a smaller burst amplitude.

We performed a DNA-trapping assay in which Pol X preincubated with labeled 45(G) DNA was mixed with dCTP and a 10-fold excess of nonlabeled 35(G) DNA. The final concentrations after mixing were as follows: 200 nM Pol X, 200 nM labeled 45(G) DNA, 2 μM nonlabeled 35(G) DNA, and 500 μM dCTP. It is important to note that Pol X binds DNA with a high affinity ($K_D^{DNA}$) in the low nanomolar range; see ref 26 and this report); therefore, most of the labeled 45(G) DNA should be in the Pol X•DNA binary complex. The product formation exhibited burst kinetics with an amplitude of 44 ± 3 nM (Figure 2), which is ~3-fold smaller than the burst amplitude in the control experiment from which nonlabeled DNA was omitted. In the second control experiment, where labeled DNA was mixed with nonlabeled DNA, the burst amplitude was 11 ± 3 nM, which closely corresponds to the percentage of labeled 45(G) DNA in the total DNA pool. These results suggest that a large fraction of the preincubated DNA dissociates from the preformed Pol X•DNA binary complex before nucleotide incorporation. This implies that either dissociation of DNA from the Pol X•DNA complex is much faster than binding of dNTP to the Pol X•DNA complex or the preformed binary complex is nonproductive and Pol X binds DNA in a productive manner only after binding to dNTP. Trapping of one-third

![Figure 2: DNA-trapping experiment under pre-steady-state conditions. Product formation as a function of time when solution A, containing 200 μM 45(G) DNA substrate and 200 μM Pol X, was mixed with solution B, containing dCTP and both labeled 45(G) DNA and nonlabeled 35(G) DNA, exhibits burst kinetics with an amplitude of 44 ± 3 nM, a burst rate constant of 0.091 ± 0.010 s$^{-1}$, and a steady-state rate of 0.39 ± 0.03 nM/s (red). A control experiment in which solution A, containing Pol X alone, was mixed with solution B, containing dCTP and both labeled 45(G) DNA and nonlabeled 35(G) DNA, results in burst kinetics with an amplitude of 11 ± 3 nM, a burst rate constant of 0.086 ± 0.051 s$^{-1}$, and a steady-state rate of 0.31 ± 0.03 nM/s (black).]

of the DNA suggests that even though free Pol X might bind DNA in a nonproductive manner, dNTP binding to the binary Pol X•DNA binary complex induces DNA rearrangement without its dissociation from the enzyme.

**Nucleotide-Trapping Assay under Pre-Steady-State Conditions.** To establish whether dNTP can bind to Pol X in a productive manner and become incorporated into the DNA
substrate without first dissociating, we performed a dNTP-trapping assay similar to the DNA-trapping assay described above. In this experiment, we utilized the ability of Pol X to incorporate 2′,3′-dideoxyguanosine 5′-triphosphate (ddGTP) with an efficiency similar to that for unmodified dGTP (data not shown). Incorporation of ddGTP into 35(CG) DNA (two-nucleotide gap DNA with 5′-CG templating bases) leads to chain termination, thus preventing incorporation of the second nucleotide (dCTP), which in turn results in accumulation of the DNA_{n+1} product.

In the nucleotide-trapping assay, Pol X, preincubated with 50 µM ddGTP, was mixed with 100 nM 35(CG) DNA, 500 µM dGTP (competing nucleotide), and 500 µM dCTP (nucleotide for the second incorporation). All concentrations listed above are postmixing. For the control reaction, ddGTP was added to solution B instead of solution A. Total (DNA_{n+1} + DNA_{n+2}) product formation (∆) shows similar kinetics as the control (○); however, accumulation of the DNA_{n+1} product was significantly higher (63 ± 2 nM) in the first reaction (◇) compared to the control (◇), where only 14 ± 1 nM DNA_{n+1} product accumulated.

**Varied Order of Substrate Mixing in Stopped-Flow Fluorescence Assays.** To further elucidate the microscopic steps underlying the unusual substrate binding order of Pol X, we investigated Pol X reactions in stopped-flow assays. First, the effect of the varied order of substrate addition was examined using 2-aminopurine-2′-deoxyribose 5′-triphosphate (2-AP-dNTP), a fluorescent analogue of dATP. Addition of 2-AP-dNTP to Pol X resulted in a relatively fast fluorescence decay (Figure 4, red trace), while addition of 2-AP-dNTP to Pol X preincubiated with 36dd(T) DNA produced a much slower fluorescence decay (Figure 4, blue trace), suggesting that dNTP binds to Pol X faster in the absence of DNA. We also measured the rate of dissociation of 2-AP-dNTP from the Pol X·2-AP-dNTP binary complex and the Pol X·2-AP-dNTP·ddDNA ternary complex as the rate of 2-AP fluorescence increase upon mixing of the preformed complexes with an excess of ddATP (data not shown). In both cases, the rates were very similar. Together, the results suggest that Pol X has a weaker binding affinity for 2-AP-dNTP in the presence of DNA. When ddDNA was added to the Pol X·2-AP-dNTP complex, a fast fluorescence decay was observed (Figure 4, first phase in the green trace), suggesting that DNA binds to the Pol X·2-AP-dNTP complex at a rate much faster than the rate of binding of 2-AP-dNTP to the Pol X·DNA complex. Note that the slow fluorescence increase seen in the green trace is due to the release of 2-AP-dNTP because of the weaker binding affinity in presence of DNA, as described above. This is also supported by decrease in the relative amplitude of this phase when all the substrate concentrations are increased to 5 µM (dark green trace).
Kinetics of Nucleotide Binding and Dissociation. To determine the kinetic parameters for Pol X–dNTP binding and dissociation, we created the H115W mutant containing a fluorescent Trp residue in the active site. WT Pol X also contains a Trp residue away from the active site, but it does not exhibit a significant fluorescence change upon nucleotide binding and incorporation (data not shown). We first compared the binding and dissociation of 2-AP-dNTP to Pol X and H115W utilizing the fluorescence signal from 2-AP. Both enzymes exhibited similar kinetics of 2-AP-dNTP binding and dissociation (Supporting Information, Figure S2A,B). Then we monitored the Trp fluorescence signal in response to 2-AP-dNTP binding. The rate of Trp fluorescence change was identical to that of the 2-AP fluorescence change of the nucleotide (Supporting Information, Figure S2C), implying that both the fluorescent probes respond to the same molecular process, namely, nucleotide binding. On the basis of the observations described above, kinetic parameters for nucleotide binding to H115W can be assumed to be similar to that for WT Pol X.

To calculate the bimolecular rate of nucleotide binding, 1 µM H115W was mixed with incremental amounts of one of four dNTPs, in the presence of 10 mM free Mg2+ in both solutions, and the rate of fluorescence change was measured and plotted as a function of nucleotide concentration (Figure 5A,B). The bimolecular rate of dNTP binding was calculated by pseudo-first-order approximation as the slope of this linear concentration dependence. We also investigated the dependence of dNTP binding to H115W on Mg2+ concentration. Our data suggest that the enzyme binds dNTP only in the form of Mg2+·dNTP (Supporting Information, Figure S3). To measure the rate of nucleotide dissociation, H115W incubated with an excess of either dNTP was mixed with EDTA in a stopped-flow apparatus and the change in fluorescence was fit to a single-exponential equation (Figure 5C). The ability of EDTA to effect dNTP dissociation was comparable to that by addition of an excess of dNTP; when the H115W-2-AP-dNTP complex was mixed with excess dATP, dCTP, dGTP, dTTP, or EDTA, the fluorescence signal changed with similar rates of 0.048, 0.053, 0.048, 0.053, and 0.069 s−1, respectively. Also, this rate was independent of the concentration of the binary complex as well as the excess dNTP concentration. Table 3 lists the kinetic parameters for binding and dissociation of each nucleotide with H115W and calculated $K^d_{dNTP}$ values. Figure 5 and Table 3 clearly show that H115W, and Pol X by implication, can bind dNTP very tightly. Also noticeable is the fact that the purine nucleotides bind faster and dissociate slower than the pyrimidine nucleotides, resulting in a 10–25-fold lower $K_d$ for the purines. $K_d$ values for dGTP and dTTP determined in these studies are in an excellent agreement with the previously published data (27). For dCTP and dATP, there is small discrepancy in determined values: $K^d_{dCTP}$ is somewhat larger than those previously published, whereas $K^d_{dTTP}$ is smaller.

Kinetics of Pol X–DNA Binding and Dissociation. Kinetics of DNA binding and dissociation were investigated using a 2-AP fluorescent probe in DNA [35dd(GA)] (Table 1) in stopped-flow assays. Binding of DNA to Pol X resulted in a fast increase in 2-AP fluorescence (Figure 6A). This fluorescence change can be best described by second-order binding kinetics with a $k_{on}$ of 774 ± 30 µM−1 s−1. To measure the rate of dissociation of the Pol X·DNA complex, Pol X was preincubated with a 2-AP-labeled DNA substrate and then rapidly mixed with a 10-fold excess of nonlabeled DNA. The observed fluorescence change exhibited single-exponential character with a $k_{off}$ of 2.38 ± 0.04 s−1 (Figure 6B).
This rate was found to be independent of the concentration of enzyme or nonlabeled DNA, supporting the proposal that the observed fluorescence change corresponds to the Pol X-DNA dissociation step. The $K_d^{DNA}$ value of 2.9 nM determined from kinetic data ($K_d^{DNA} = k_{off}/k_{on}$) correlates very well with the values of 3 nM obtained from an EMSA (26) but is somewhat higher than $K_d$ values (0.017–1.5 nM) obtained from quantitative fluorescence studies (28).

Further Insights into Preferred Binding Order Provided by Stopped-Flow Fluorescence Assays with a 2-AP Probe in DNA Substrate. The effect of the order of substrate mixing was also investigated in stopped-flow assays using 18dd/35AP DNA (Table 1). Pol X readily accepts this DNA substrate, and the template–primer substrate with a 2-AP modification at the +1 templating position has been previously found to provide the best signal-to-noise ratio in stopped-flow fluorescence assays (35, 36). Addition of dCTP to the Pol X·ddDNA binary complex exhibited a slow fluorescence change with a single-exponential rate of 1.64 s$^{-1}$ (blue trace in Figure 7A), whereas addition of DNA to a preformed Pol X·dATP complex induced a much faster fluorescence change which fit to a double-exponential equation with rates of 79.3 and 9.92 s$^{-1}$ (red trace in Figure 7A). These observations suggest that preceding dNTP binding facilitates following DNA binding and/or rearrangements that lead to a kinetically active ternary complex. To understand the reasons for a slower fluorescence change upon dNTP binding to a preformed Pol X·DNA binary complex,
we analyzed the effect of dCTP concentration on the rate of this fluorescence change (Supporting Information, Figure S4A). We found that the rate increases linearly with an increase in dCTP concentration with a slope equal to 0.0023 µM⁻¹ s⁻¹ (Supporting Information, Figure S4B). Comparison of this value with the second-order rate constant for dCTP binding to free Pol X [0.055 µM⁻¹ s⁻¹ (Table 3)] implies that preceding DNA binding hinders subsequent nucleotide binding, which in turn results in the nucleotide binding being the rate-limiting step during the formation of the Pol X·DNA·dNTP ternary complex.

We then investigated how the presence of mismatched substrates would influence the appearance of stopped-flow fluorescence traces. Addition of 500 µM dATP to Pol X preincubated with 18dd/35AP DNA resulted in a fluorescence change in the direction opposite of that of the fluorescence change observed when dCTP was used (blue trace in Figure 7B). At the same time, addition of DNA substrate to Pol X preincubated with dATP did not produce a significant change in fluorescence intensity (red trace in Figure 7B). The small fluorescence increase might be attributed to the formation of a mismatched ternary complex or the formation of a small fraction of the Pol X·DNA binary complex. The latter suggestion is more plausible because it was found that the amplitude of the fluorescence change decreased at higher concentrations of dATP (data not shown). These results are consistent with the proposal that 18dd/35AP DNA with dG in a templating position cannot efficiently bind to the preformed Pol X·dATP binary complex. Accordingly, addition of dATP to the preformed Pol X·DNA complex induces DNA dissociation which results in a decrease in fluorescence (hence, the opposite direction of fluorescence change). This hypothesis is also consistent with the observation that the addition of a 10-fold molar excess of nonlabeled DNA to Pol X preincubated with dATP and 18dd/35AP DNA did not cause a significant fluorescence change, implying that most of the 2-AP-labeled DNA was in the unbound state (green trace in Figure 7B). Stopped-flow results are also in excellent agreement with the conclusion derived from the steady-state inhibition assays that the ddDNA substrate analogue with a mismatch at the templating position has a lower affinity for the Pol X·dNTP binary complex.

**DISCUSSION**

A number of DNA polymerases have been discovered in recent years. Accordingly, a number of structural and functional studies have been performed to improve our understanding of the mechanisms underlying their novel (and often unusual) properties (see refs 19 and 20 for reviews). For most of these DNA polymerases, kinetic analyses have been conducted using schemes and methodology developed earlier in classical studies of high-fidelity DNA polymerases. For example, pre-steady-state kinetic experiments are generally designed with the assumption that the DNA polymerases initially form a binary complex with a DNA substrate and then bind a nucleotide substrate (10–12, 15, 21). The report presented here is the first analysis of the substrate binding order performed for a low-fidelity DNA polymerase. Our findings imply that different DNA polymerases might display different preferences for the order in which the two substrates bind.

In this study, we have employed classical approaches, such as steady-state inhibition assays and DNA-trapping assays, as well as novel experimental designs, for instance, use of dATP as an inhibitor and dNTP-trapping assays, to elucidate the order of DNA and dNTP substrate binding by Pol X. Our observations of uncompetitive inhibition by the ddDNA analogue when dNTP substrate was varied and noncompetitive inhibition by noncognate dNTP when the DNA substrate was varied are not consistent with the strictly ordered sequential mechanism with DNA as the first binding substrate. Our results can be interpreted only using a scheme in which dNTP binding is required for kinetically productive DNA binding. We should note that when the ddDNA analogue with a mismatch at the templating position was used, inhibition pattern deviated from uncompetitive behavior, implying that Pol X can bind DNA utilizing an alternative, dNTP-independent binding mode. Nevertheless, our results strongly suggest that dNTP is the preferred first substrate in the Pol X kinetic mechanism. These conclusions were also supported by substrate-trapping experiments under pre-steady-state conditions. Our results show that preincubated dNTP is preferably incorporated by Pol X, whereas Pol X·DNA trapping is not efficient. These data show that Pol X can bind a nucleotide substrate in a productive manner in the absence of DNA substrate, while DNA binding to Pol X, in the absence of dNTP, is mostly nonproductive. It suggests that, during the formation of a catalytically competent ternary complex, Pol X preferably binds DNA as the second substrate. Our proposed substrate binding order is also consistent with the highly distributive nature of polymerization by Pol X (22).

It is known that many DNA polymerases are able to bind dNTP in the absence of DNA substrate, forming a nonproductive E·dNTP binary complex. For example, X-ray structural studies of Klenow fragment of *E. coli* Pol I suggest that, in the absence of DNA substrate, the base and sugar moieties of bound dNTP adopt multiple conformations (6). Also, the structural analysis of mammalian Pol β revealed that dNTP binding interactions with this polymerase differ drastically between binary Pol β·dNTP and ternary Pol β·DNA·dNTP complexes (7, 33). While the incoming nucleotide makes a number of contacts with the active site residues in the ternary complex, the base of dNTP found in the crystal structure of the Pol β·dNTP binary complex does not interact with the active site and is directed toward residues from another asymmetric unit. The only structural moiety that remains similarly positioned in both the structures is γ-phosphate. NMR spectroscopy of methionine-labeled Pol β indicates that, in contrast to the observed global chemical shift perturbations upon formation of the Pol β·DNA·dNTP ternary complex, binding of dNTP alone does not cause global changes in the NMR spectra, suggesting that the enzyme structure remains largely unchanged. Interestingly, the increased complexity observed in these spectra may be attributed to the existence of multiple conformational states of the binary Pol β·dNTP complex (39).

The studies presented here show that Pol X binds dNTP very tightly and productively. Addressing the effect of DNA on the kinetics of dNTP binding and dissociation, we found that while the rate of dNTP dissociation was not affected by the presence of DNA, the rate of formation of the ternary complex was significantly reduced when Pol X was prein-
cubated with a DNA substrate. One possible explanation for this observation is that DNA binding by Pol X prior to dNTP binding brings the enzyme into a nonproductive conformation where subsequent nucleotide substrate binding is hindered or the rearrangement of the nonproductive ternary complex into its active conformation is retarded. On the other hand, preincubation of Pol X with a dNTP substrate might bring the enzyme into a conformation where productive DNA binding is facilitated while nonproductive DNA binding is prevented. This hypothesis is supported by the observation that the rate of DNA conformational rearrangement in the Pol X-DNA-dNTP ternary complex, as determined in 2-AP fluorescence stopped-flow assays, depends on the order of substrate mixing. In agreement with conclusions from our results, structural NMR studies of Pol X have shown that purine dNTP binding to the free enzyme induces large $^{1}H$ and $^{15}N$ chemical shift perturbations, which likely reflect a global change in Pol X conformation (27). It is important to note that nucleotide binding induces large chemical shift perturbations in residues in helices αC and αE, the proposed DNA-binding site in Pol X. It is plausible that nucleotide binding and the following conformational change create a binding site for the DNA substrate with a complementary nature of nucleotide incorporation catalyzed by DNA polymerases. Additionally, as processive enzymes, DNA polymerases should remain bound to DNA substrate to carry out processive polymerization, which also implies an ordered mechanism with DNA as the leading substrate. This proposed mechanism likely remains true for “classical” DNA polymerases. However, a number of recently discovered DNA polymerases with novel properties (20) might not have to follow the same presumed rationales. For instance, processivity is not a universally required feature of all DNA polymerases since most repair DNA polymerases, such as lesion bypass or BER polymerases, have evolved to incorporate a single dNTP at the site of DNA damage. While processivity necessitates a “DNA-first” order of substrate binding, a purely distributive polymerase would possibly be capable of binding dNTP as the first substrate. Even though an ordered “dNTP-first” binding mechanism would not be logical for mildly processive polymerases, they may still deviate from the classical DNA-first ordered mechanism and could, theoretically, display random sequential substrate binding. In addition to the lack of significant processivity, the determinative role of the templating base in the selection of incoming nucleotide could also be greatly reduced for low-fidelity DNA polymerases. A remarkable example of the weakened influence of the template can be found in Rev1, a member of DNA polymerase family Y, which utilizes a unique mechanism of nucleotide selection. This enzyme preferentially binds and incorporates dCTP regardless of the templating base (17, 44, 45). The crystal structure of the Rev1 ternary complex revealed that incoming nucleotide selection is dictated by the interaction of dCTP with an Arg324 residue, rather than base pairing with a dG template (46). Pol η, a low-fidelity DNA polymerase from family Y, can serve as another example of the reduced role of the DNA template in dNTP binding. As found in a recent crystal structure of the ternary complex of Pol η with the correct nucleotide and DNA substrate containing a cisplatin-induced lesion (47), the enzyme is able to preorient the incoming nucleotide and two metal ions in the active conformation even if the templating nucleotide is positioned outside the active site. Again, it has been suggested that the correct positioning of the incoming dNTP in the active site is mainly realized via interaction with the protein residues (47).

Similar to the above-mentioned low-fidelity polymerases, Pol X could also employ protein-mediated dNTP selection. For example, preferential affinity toward purine nucleotides could result from specific interactions with the active site residues. The structure of Pol X in complex with its substrates, which is not available at present, could improve our understanding of this substrate preference and, perhaps, of the altered order of substrate binding.

One could ask how general this altered mechanism is among other DNA polymerases. It would be too hasty to reach a definite conclusion that the altered binding order is universal to any extent. However, having Pol X as a precedent, we can conclude with certainty that this mechanism is possible for other DNA polymerases as well. Lesion bypass and repair polymerases could be potential candidates. Nucleotide prebinding might assist in the recognition of specific sites of DNA damage and facilitate their accurate bypass and/or repair. Overall, the findings of the studies presented here provide new directions toward an understanding of how DNA polymerases might employ distinct mechanisms to aid in their biological functions.
42. Werneburg, B. G., Ahn, J., Zhong, X., Hondal, R. J., Kraynov, V. S., and Tsai, M.-D. (1996) DNA Polymerase β: Pre-Steady-State Kinetic Analysis and Roles of Arginine-283 in Catalysis and Fidelity. *Biochemistry* 35, 7041–7050.

43. Jezewska, M. J., Bujalowski, P. J., and Bujalowski, W. (2007) Interactions of the DNA polymerase X of African swine fever virus with double-stranded DNA. Functional structure of the complex. *J. Mol. Biol.* 373, 75–95.

44. Nelson, J. R., Lawrence, C. W., and Hinkle, D. C. (1996) Deoxycytidyl transferase activity of yeast REV1 protein. *Nature* 382, 729–731.

45. Washington, M. T., Minko, I. G., Johnson, R. E., Haracska, L., Harris, T. M., Lloyd, R. S., Prakash, S., and Prakash, L. (2004) Efficient and Error-Free Replication past a Minor-Groove N2-Guanine Adduct by the Sequential Action of Yeast Rev1 and DNA Polymerase ζ. *Mol. Cell. Biol.* 24, 6900–6906.

46. Nair, D. T., Johnson, R. E., Prakash, L., Prakash, S., and Aggarwal, A. K. (2005) Rev1 Employs a Novel Mechanism of DNA Synthesis Using a Protein Template. *Science* 309, 2219–2222.

47. Alt, A., Lammens, K., Chiocchini, C., Lammens, A., Pieck, J. C., Kuch, D., Hopfner, K.-P., and Carell, T. (2007) Bypass of DNA lesions generated during anticancer treatment with cisplatin by DNA polymerase η. *Science* 318, 967–970.