



Kinetic mechanism of the ssDNA recognition by the polymerase X from African Swine Fever Virus. Dynamics and energetics of intermediate formations [☆]

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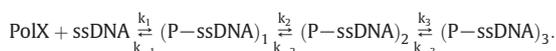
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ABSTRACT

Kinetic mechanism of the ssDNA recognition by the polymerase X of African Swine Fever Virus (ASFV) and energetics of intermediate formations have been examined, using the fluorescence stopped-flow method. The association is a minimum three-step process



The nucleic acid makes the initial contact through the C-terminal domain, which generates most of the overall ΔG° . In the second step the nucleic acid engages the N-terminal domain, assuming the bent structure. In equilibrium, the complex exists in at least two different states. Apparent enthalpy and entropy changes, characterizing formations of intermediates, reflect association of the DNA with the C-terminal domain and gradual engagement of the catalytic domain by the nucleic acid. The intrinsic DNA-binding steps are entropy-driven processes accompanied by the net release of water molecules. The final conformational transition of the complex does not involve any large changes of the DNA topology, or the net release of the water molecules.

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1. Introduction

The African Swine Fever Virus (ASFV) is the etiological agent responsible for acute hemorrhagic fever of domestic pigs (see accompanying paper) [1–6]. Besides the replicative DNA polymerase, the DNA genome of the virus encodes another ~20 kDa DNA polymerase, a member of the pol X family referred to as the ASFV pol X [1–3]. The major physiological role of the ASFV pol X is to repair the damaged viral DNA [5,6]. The pol X family comprises several polymerases with different and specialized functions in the cell [7–9]. The well-known member of the family is the mammalian pol β , which plays a very specialized function in the DNA repair processes [8–12]. The structure of pol β shows a typical polymerase fold, containing a thumb, palm, and fingers domains, due to its resemblance to the human hand [12–16]. It also possesses an additional, N-terminal 8-kDa domain, which is the primary DNA-binding site of the enzyme with a significant

degree of functional autonomy and spatial separation from the rest of the enzyme molecule [13–17].

The NMR studies of ASFV pol X revealed a structure, which is very different from pol β and other DNA replicative or repair polymerases [18,19]. The enzyme is built only of the N-terminal domain, which includes the first 105 amino acids from the N-terminus of the protein and the C-terminal domain, which encompasses the remaining 69 amino acid residues (see accompanying paper) [18,19]. Both the N-terminal domain and the C-terminal domain contains highly positively charged helices, αC and αE , respectively, which do not have their counterparts in pol β . Nevertheless, the entire molecule of the ASFV pol X corresponds to the palm domain of the typical DNA replicative or repair polymerase. Nevertheless, in spite of the simplified structure, thermodynamic studies have already shown very complex characteristics of the ASFV pol X interactions with the ssDNA (see accompanying paper) [20–22].

The total DNA-binding site of the enzyme encompasses 16 ± 1 nucleotides and possesses a heterogeneous structure, which includes the strong and the weak ssDNA-binding subsites (see accompanying paper) [20–22]. The strong ssDNA-binding subsite engages only 7 ± 1 nucleotides and generates the predominant part of the free energy of binding. Studies described in the accompanying paper indicated that the strong DNA-binding subsite is the primary DNA-binding site of the enzyme, independent of the nucleic acid conformation and is located on

Abbreviations: ASFV, African Swine Fever Virus; DTT, dithiothreitol; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ϵA , etheno-adenosine; CP, 7-Diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin; FRET, fluorescence resonance energy transfer.

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the C-terminal domain of the enzyme. With the longer DNA oligomers, the enzyme additionally engages the weak DNA-binding subsite on the catalytic N-terminal domain in interactions with the nucleic acid, although the efficiency of the engagement depends on the length of the nucleic acid [20]. The intrinsic affinity of the total DNA-binding site is not a simple sum of contributions from the intrinsic affinities of strong and the weak DNA-binding subsites. Moreover, the affinities of the subsites differ by at least two orders of magnitude. This large affinity difference results from specific conformational changes, induced by the engagement of the weak DNA-binding subsite in interactions with the DNA and characterized by a large positive enthalpy change. The intrinsic affinities of the strong DNA-binding subsite and the total DNA-binding site are mostly driven by large entropy changes, predominantly resulting from the release of water molecules (see accompanying paper).

Elucidation of the kinetics of the ASFV pol X association with the nucleic acid is a prerequisite for understanding the recognition processes of the nucleic acid. This is of particular importance in the case of the DNA repair polymerases, which must recognize specific structure of the damaged DNA. Moreover, dynamics of the DNA recognition are of paramount significance for understanding the dynamics of DNA synthesis, as the recognition processes precede and determine the overall efficiency of the catalysis. In spite of the importance of the ASFV pol X in the DNA metabolism of the ASFV virus, the kinetic mechanisms of the enzyme interactions with the ssDNA, as well as the energetics of the intermediate formations, have never been quantitatively addressed.

In this communication, we examine the kinetic mechanisms of interactions of the strong DNA-binding subsite and the total DNA-binding site of the ASFV pol X with the ssDNA, and energetics of the intermediate formations. The DNA makes initial contact through the strong DNA-binding subsite. The first binding step is very close to the diffusion-controlled reaction, although it generates most of the overall ΔG° of binding. The second step is also very fast and occurs within ~ 1 ms of the reaction time. The DNA engages the weak DNA-binding subsite and the bound nucleic acid assumes the bent structure. In equilibrium, the polymerase–ssDNA complex exists in at least two different states. The intrinsic DNA-binding steps to the strong and the weak subsites are entropy-driven processes and accompanied by the release of similar numbers of water molecules from each subsite.

2. Materials and methods

2.1. Reagents and buffers

All solutions were made with distilled and deionized >18 MW (Milli-Q Plus) water. All chemicals were reagent grade. Buffer C is 10 mM sodium cacodylate adjusted to pH 7.0 with HCl, 1 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 10% glycerol (w/v). The temperature in the buffer is indicated in the text.

2.2. ASFV Pol X

Isolation and purification of the protein was performed as previously described (see accompanying paper) [20–22].

2.3. Nucleic acids

All nucleic acids were purchased from Midland Certified Reagents (Midland, Texas). Concentrations of all ssDNA oligomers have been spectrophotometrically determined, as previously described by us [20–25].

2.4. Stopped-flow kinetics

All fluorescence stopped-flow kinetic experiments were performed using SX.MV18 stopped-flow instrument (Applied Photophysics Ltd.

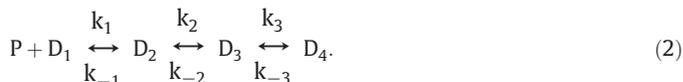
Leatherhead, UK) [26–35]. The reactions were monitored using the fluorescence of the etheno-derivatives of the ssDNA oligomers, $d\epsilon(p\epsilon A)_9$ and $d\epsilon A(p\epsilon A)_{19}$, with $\lambda_{ex} = 325$ nm, or, in the case of 5'Fl-dT (pT)₁₉-CP-3', with $\lambda_{ex} = 425$ nm, with the excitation monochromator slits at 1 mm (band pass ~ 4.5 nm). The emission intensity was observed through the emission monochromator set at 410 nm or 520 nm, for the etheno-derivatives and 5'Fl-dT(pT)₁₉-CP-3' respectively. Usually, 11–15 traces were collected and averaged for each sample [26–35]. The kinetic curves were fitted to extract relaxation times and corresponding amplitudes, using nonlinear least-squares software provided by the manufacturer, with the exponential function defined as

$$F(t) = F(\infty) + \sum_{i=1}^n A_i \exp(-\lambda_i t) \quad (1)$$

where $F(t)$ is the fluorescence intensity at time t , $F(\infty)$ is the fluorescence intensity at $t = \infty$, A_i is the amplitude corresponding to i th relaxation process, λ_i is the time constant (reciprocal relaxation time) characterizing i th relaxation process, and n is number of relaxation processes. All analyses of the data were performed using Mathematica (Wolfram, Urbana, IL) and Kaleida Graph (Synergy Software, PA) [26–35].

2.5. Analysis of stopped-flow kinetic experiments

Quantitative examinations of both the relaxation times and the amplitudes of the observed kinetic processes have been performed using the matrix projection operator approach, as previously described by us [26–35]. Briefly, the most complex mechanism of the ASFV pol X (P) binding to the ssDNA (D), which encompass the total DNA-binding site of the enzyme, includes the bimolecular step, which is followed by two, first-order conformational transitions as



The reaction is monitored by the fluorescence change of the DNA. Under pseudo-first-order conditions, the total concentration of the protein is much larger than the total concentration of the nucleic acid, i.e., $[P]_T \gg [D]_T$, the time-dependence of different nucleic acid species is expressed, using matrix projection operators, \mathbf{Q}_i , as [26,27,36,37]

$$\mathbf{N} = \mathbf{Q}_0 \mathbf{N}_0 + \mathbf{Q}_1 \mathbf{N}_0 \exp(\lambda_1 t) + \mathbf{Q}_2 \mathbf{N}_0 \exp(\lambda_2 t) + \mathbf{Q}_3 \mathbf{N}_0 \exp(\lambda_3 t) \quad (3)$$

where λ_1 , λ_2 , and λ_3 are eigenvalues of coefficient matrix, \mathbf{M} , of the reaction, and \mathbf{N}_0 is the vector of initial concentrations. The projection operators, \mathbf{Q}_i , are defined, using the original coefficient matrix \mathbf{M} of the reaction and its eigenvalues, λ_i as

$$\mathbf{Q}_i = \frac{\prod_{j \neq i} (\mathbf{M} - \lambda_j \mathbf{I})}{\prod_{j \neq i} (\lambda_i - \lambda_j)} \quad (4)$$

where n is the number of eigenvalues and \mathbf{I} is the identity matrix. In the considered reaction, there are four eigenvalues λ_0 , λ_1 , λ_2 , and λ_3 , with $\lambda_0 = 0$ because of the mass conservation in the system. Eq. (3) becomes

$$\begin{pmatrix} D_1 \\ D_2 \\ D_3 \\ D_4 \end{pmatrix} = \begin{pmatrix} P_{01} \\ P_{02} \\ P_{03} \\ P_{04} \end{pmatrix} + \begin{pmatrix} P_{11} \\ P_{12} \\ P_{13} \\ P_{14} \end{pmatrix} \exp(\lambda_1 t) + \begin{pmatrix} P_{21} \\ P_{22} \\ P_{23} \\ P_{24} \end{pmatrix} \exp(\lambda_2 t) + \begin{pmatrix} P_{31} \\ P_{32} \\ P_{33} \\ P_{34} \end{pmatrix} \exp(\lambda_3 t) \quad (5)$$

where P_{ij} is the j th element of the projection of the vector of the initial concentrations \mathbf{N}_0 on the eigenvector, corresponding to the i th

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