Watching the Bacteriophage N4 RNA Polymerase Transcription by Time-dependent Soak-trigger-freeze X-ray Crystallography*

Received for publication, June 4, 2012, and in revised form, November 29, 2012. Published, JBC Papers in Press, December 12, 2012, DOI 10.1074/jbc.M112.387712

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The challenge for structural biology is to understand atomic-level macromolecular motions during enzymatic reaction. X-ray crystallography can reveal high resolution structures; however, one perceived limitation is that it reveals only static views. Here we use time-dependent soak-trigger-freeze X-ray crystallography, namely, soaking nucleotide and divalent metal into the bacteriophage RNA polymerase (RNAP)-promoter DNA complex crystals to trigger the nucleotidyl transfer reaction and freezing crystals at different time points, to capture real-time intermediates in the pathway of transcription. In each crystal structure, different intensities and shapes of electron density maps corresponding to the nucleotide and metal were revealed at the RNAP active site which allow watching the nucleotide and metal bindings and the phosphodiester bond formation in a time perspective. Our study provides the temporal order of substrate assembly and metal co-factor binding at the active site of enzyme which completes our understanding of the two-metal-ion mechanism and fidelity mechanism in single-subunit RNAPs. The nucleotide-binding metal (MeB) is coordinated at the active site prior to the catalytic metal (MeA). MeB coordination is only temporal, established just before and dissociated immediately after phosphodiester bond formation. We captured these elusive intermediates exploiting the slow enzymatic reaction in crystals. These results demonstrate that the simple time-dependent soak-trigger-freeze X-ray crystallography offers a direct means for monitoring enzymatic reactions.

The phosphoryl transfer reactions including the nucleotidyl transfer play a fundamental role in genome maintenance and gene expression (6, 7). Nucleotidyl transferases, DNA polymerase (DNAP) and RNA polymerase, catalyze the nucleophilic attack of the 3′-O oxygen of the primer terminus onto the 5′-α-phosphate of the incoming nucleotide. The reaction proceeds via a “two-metal-ion mechanism” involving two or three conserved aspartates that position two divalent cations in the active site; a catalytic metal (MeA) is a Lewis acid to reduce the pKa of the primer 3′-OH bound at the P-site (+1 nucleotide in this study, Fig. 1d) whereas a nucleotide-binding metal (MeB) plays roles in coordinating the triphosphate groups of the nucleotide at the N-site (+2 nucleotide in this study, Fig. 1d) and in stabilizing the pentacovalent phosphate intermediate (4–6, 8). These enzymes are characterized by the flexibility of substrate utilization: DNAP uses four different deoxynucleotide triphosphates, and RNAP uses four different nucleotide triphosphates, combined with DNA sequence-dependent correct nucleotide addition (9, 10).

Stopped-flow kinetics (9–12) and x-ray crystallographic studies of the A-family polymerases, including bacterial DNAP I Klenow fragment (13, 14) and bacteriophage DNAP (15) and RNAPs (16, 17), have elucidated the kinetic scheme of the nucleotide addition cycle and provided structural perspectives on nucleotide incorporation and selection. These studies proposed the presence of at least two prechemistry steps as the nucleotide selectivity checkpoints, including conformational change of the Fingers O-helix and another unidentified step preceding phosphodiester bond formation (9, 10).

One of the strategies for trapping elusive intermediate of nucleic acid polymerases is the use of a 3′-deoxynucleotide analog (13–15, 18), which lacks the essential O3′-nucleophile for the phosphodiester bond formation. The intermediate struc-

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*This work was supported, in whole or in part, by National Institutes of Health Grant AI12575.

The atomic coordinates and structure factors (codes 4FF1, 4FF2, 4FF3, and 4FF4) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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tures captured by using this nucleotide analog revealed the rotation of Fingers subdomain including the O-helix, which is known as the “closing of Fingers,” upon nucleotide binding at the active site and proposed that the DNA rearrangement plus the closing of Fingers is the early fidelity checkpoint of the nucleotide selection. However, the 3’-deoxynucleotide analog is not able to coordinate MeA, resulting in misalignment of the reactive groups. Another actively used substrate analog is the nonreactive NMPCPP, which allows the coordination of the critical MeA but prevents the phosphodiester bond formation due to the nonhydrolyzable methylene substitution on its triphosphate. Using this analog, intermediates in the T7 RNAP elongation and bacteriophage N4 RNAP initiation pathways were trapped and revealed the prechemistry events (16, 17, 19). These structures showed that substrate and metal binding induced the closing of the Fingers to engage basic residues on the O-helix to interact with the incoming nucleotide triphosphates and thus provided the structural basis of the primary substrate selection mechanism. Our recent studies of transcript initiation by the central domain of N4 phage virion-encapsulated RNAP (mini-vRNAP), thus presented the substrate complex (SCI) and a postchemistry product complex (PC), which provide a complete set of snapshots during the transcript initiation process from the nucleotide and metal bindings to the phosphodiester bond formation (Fig. 1). Transition from a binary RNAP-promoter DNA complex (BC) to the ternary SCI mainly involved the closing of Fingers and template DNA rearrangement. Although the closing of Fingers is a common early conformational change seen in all A-family polymerases upon substrate binding, the extent of movements is not the same in all. The rotation of the Fingers is more modest in T7 RNAP (16, 20) with respect to DNAP I (14, 15) and further subtle in the N4 mini-vRNAP (17). Comparison of the two precatalytic intermediates SCI and SCII identified critical realignment of the substrates to a catalysis-competent conformation brought about by the addition of catalytic MeA in SCII. We thus proposed that the MeA binding is the last step before the phosphodiester bond formation. The same conclusion was also proposed by the stopped-flow kinetics of DNAP I (11). The kinetic scheme of the same N4 mini-vRNAP transcript initiation process in crystalllo was investigated by Raman crystallography and revealed that the DNA rearrangement and the closing of Fingers are completed immediately after soaking nucleotide and metal into RNAP-DNA complex crystals whereas the metal binding and phosphodiester bond formation occurred at a later stage of the reaction (21). This work showed that the enzyme remains active in crystalllo and found that the single-turnover nucleotidyl transfer reaction in crystalllo proceeds substantially slower (sec-
TABLE 1
Data collection and refinement statistics of the N4 mini-vRNAP transcript initiation complexes
Data sets were collected at MacCHESS-F1, Ithaca, NY. Highest resolution shell is shown in parentheses.

| Complex          | TIC-1’        | TIC-2’        | TIC-3’        | TIC-4’        |
|------------------|---------------|---------------|---------------|---------------|
| Crystal parameters | PDB code      | PDB code      | PDB code      | PDB code      |
|                   | 4FF1          | 4FF2          | 4FF3          | 4FF4          |
| Space group       | P2_12_2_1     | P2_12_2_1     | P2_12_2_1     | P2_12_2_1     |
| Cell dimensions   |               |               |               |               |
| a (Å)             | 82.163        | 81.908        | 82.019        | 82.398        |
| b (Å)             | 111.500       | 111.457       | 111.652       | 111.779       |
| c (Å)             | 277.103       | 275.760       | 276.145       | 276.894       |
| Data collection   |               |               |               |               |
| Resolution (Å)    | 50–2.46       | 50–2.00       | 50–2.00       | 50–2.00       |
| Rmerge (%)        | 0.116 (0.426) | 0.186 (0.806) | 0.105 (0.546) | 0.127 (0.583) |
| completeness (%)  | 15.6 (2.9)    | 7.4 (1.2)     | 15.8 (2.6)    | 11.2 (1.4)    |
| Redundancy (%)    | 5.0 (3.6)     | 4.4 (3.4)     | 3.8 (3.7)     | 3.3 (2.4)     |
| Refinement        |               |               |               |               |
| Resolution (Å)    | 47.8–2.46     | 47.7–2.00     | 42.4–2.00     | 43.9–2.03     |
| No. reflections   | 87,771        | 154,842       | 153,225       | 156,753       |
| Rwork/Rfree (%)   | 15.3/21.1     | 20.8/25.5     | 18.4/21.6     | 19.1/23.9     |
| Root mean square deviations | 0.007 | 0.004 | 0.004 | 0.006 |
| Bond length (Å)   | 1.075         | 0.831         | 0.820         | 0.929         |

RESULTS

The BC crystal contains N4 mini-vRNAP bound to its promoter DNA (P2_7c, 36 bases, Fig. 1c), which has the template DNA bases of C(+1) and T(+1). Additon of GTP and ATP plus divalent cations allows formation of the transcript initiation complex (TIC) accommodating GTP at +1 position (P-site) and ATP at +2 position (N-site) that, upon phosphodieste

EXPERIMENTAL PROCEDURES

Transcript initiation complexes were prepared as follows. BC crystals were sequentially transferred through cryoprotectant solutions containing the mother liquor with increasing concentrations of PEG 3350 (30, 35, and 40%). Then crystals were transferred to the soak solution containing 5 mM GTP, 5 mM ATP, and 10 mM MnCl2 in addition to the final cryosolution composition. Crystals were harvested from the soaking solution at specific time points and flash frozen in liquid nitrogen.

Crystallographic data were processed with HKL2000 (22) (Table 1). The BC structure (23) was used as an initial model for the rigid body and TLS refinements by using Phenix (24). Nucleotides were modeled into the respective electron density maps by Coot (25). Final coordinates and structure factors were submitted to the Protein Data Bank; IDs codes are listed in Table 1.

Final concentration, c, of the N4 phage polymerase-DNA complex in a single crystal was calculated using Equation 1,

\[ c = n/(\nu \times N_a) \]  

where \( n \) is the number of molecules of complex per unit cell of the crystal; \( \nu \) is the volume of a unit cell, and \( N_a \) is Avogadro’s number. The BC crystals contain two RNA-P-DNA complexes per asymmetric unit, and in the P2_12_2_1 space group each unit cell has four asymmetric units. Thus there are \( n = 8 \) molecules/unit cell. From the unit cell parameters (82 Å × 112 Å × 276 Å), \( \nu \) is calculated to be 2.54 × 10^{-12} nl. Solving the Equation 1 concentration of complex in crystal, \( c = 5.1 \) m. The volume, \( V \), of a typical crystal was calculated from its dimensions (25 μm × 100 μm × 125 μm) = 0.3125 × 10^{6} μm^3 = 0.31 nl.
template from −1 to +2 positions are not solid (Fig. 3b) and were also observed in the BC structure (Fig. 3a), and the B-factor of the Y-678 side chain is substantially high (59.5), suggesting ongoing conformation changes of the O-helix.

At 2 min (TIC-2’, Fig. 2c), the electron density maps for Y-678 and DNA template are well ordered (Fig. 3c), and the Y-678 side chain B-factor is substantially low (22.6), indicating completion of the O-helix movement (Fig. 2g). Three Fingers
residues, Y-612, R-666, and K-670, which play a role in the +2 nucleotide binding also change their positions (Fig. 2g). Concomitantly, continuous and complete $F_o - F_c$ maps corresponding to GTP(+1) and ATP(+2) are observed. The electron densities for the $\beta$ and $\gamma$ phosphate groups of GTP(+1) are not well resolved due to their flexibility; therefore, GMP was modeled at the +1 position. In addition to nucleotides, a strong $F_o - F_c$ map for MeA is found at the active site. The $F_o - F_c$ map around the MeA binding site is weaker than the one for MeB, indicating the presence of Mn$^{2+}$ with partial occupancy. In a previous study, we found that the Mn$^{2+}$ coordinating D-559 changes its conformation upon MeA binding (17). Accordingly, the D-559 electron density map of TIC-2' (Fig. 3f) is distinct from the ones observed from SCI (coordinating MeB, Fig. 3d) and SCII (coordinating MeA and MeB, Fig. 3e) (17), suggesting the presence of alternative conformers of the D-559 side chain and supporting the presence of Mn$^{2+}$ with partial occupancy. After 2 min of soaking, some molecules contain GTP(+1), ATP(+2), and MeB corresponding to the SCI structure, and some molecules contain GTP(+1), ATP(+2), MeA and MeB corresponding to the SCII structure determined in our previous study (Fig. 1a) (17). TIC-2' is an intermediate where the two nucleotides and MeB are loaded at the active site and it is waiting for the MeA binding to form the catalytically competent complex.

At 3 min (TIC-3', Fig. 2d), a strong $F_o - F_c$ map for MeA appears at the active site, and its intensity is equivalent to the one of MeB. Except the D-559 side chain orientation, loading of MeA does not trigger any other conformational change of RNAP and DNA (Fig. 2h). After 3 min of soaking, the active site contains GTP(+1), ATP(+2), MeA and MeB, corresponding to the SCII isolated in our previous study (Fig. 1a) (17). However, in this study, we solved this elusive intermediate structure with natural substrate. TIC-3' is a fully reactive, naturally existing enzyme-substrate-cofactor complex, the closest to the pentacovalent phosphate intermediate during the nucleotidyl transfer reaction.

At 4 min (TIC-4', Fig. 2e), a strong $F_o - F_c$ map for the +1 and +2 nucleotides is found, but there is continuous density between O3'(+1) and $\alpha$P(+2) and a gap between the $\alpha$P(+2) and $\beta$P(+2) groups, indicating that this map corresponds to the 2-mer RNA (5'-cppGpA-3'). The pyrophosphate (Pp) is still coordinated by residues R-666, K-670, and Y-612 and is observed with MeB; however, the $F_o - F_c$ map for MeA is absent, indicating that the MeA dissociates from the active site immediately after phosphodiester bond formation. Accordingly, D-559 rotates its side chain (Fig. 2i) to approximately the same position found in the BC and SCII structures, indicating its coordination to single MeB (17). After 4 min of soaking, the active site contains the nascent 2-mer RNA, Pp, and MeB, corresponding to the PC isolated in our previous study (Fig. 1a) (17). The high B-factors for Pp (82.1) and MeB (92.1) suggest their dissociation from the active site.

**DISCUSSION**

In this study, we successfully captured four intermediate structures, from the nucleotide/metal bindings at the active site to the phosphodiester bond formation, of the N4 phage vRNAP transcript initiation (Figs. 1a and 2). Nucleotide soaking triggers the O-helix closing at early stage of reaction. After 1 min of soaking (TIC-1', Fig. 2b), a weak but distinct $F_o - F_c$ map for MeA is observed with fragmented +2 nucleotide electron density supporting a widely accepted but unproved prediction that MeA comes to the active site with nucleotide. After 2 min of soaking (TIC-2', Fig. 2c), both +1 and +2 nucleotides as well as MeB bindings are completed but not MeA, which is only completed at 3 min of soaking (TIC-3', Fig. 2d). This is a clear proof that MeA is loaded to the active site prior to MeA, which is the penultimate step before phosphodiester bond formation. The presence of MeA at the active site is only temporal during the reaction. After 4 min of soaking (TIC-4', Fig. 2e), the active site possesses a 2-mer RNA, Pp, and MeB but not MeA, indicating that MeA dissociates from the active site immediately after the bond formation. Therefore, leaving MeA from the active site is the first step after bond formation and is preparing for a next round of reaction. Only a temporal presence of MeA at the active site is beneficial to prevent a reverse reaction pyrophosphorysis.

We established a kinetic scheme of N4 vRNAP transcript initiation (Fig. 1a) by using a soak-trigger-freeze x-ray crystallography experiment (Fig. 2a). The kinetic scheme is based on structural snapshots from multiple crystals; however, this scheme reflects the temporal order of events in single crystal because it is consistent with results from a Raman crystallography experiment, which uses a single crystal to trace the entire reaction. Furthermore, our scheme consists of the one proposed by the fluorescence-based assays and stopped flow kinetics for *Escherichia coli* DNAP I (5-11).

Why does MeA come to the active site just before the bond formation? The MeA is a key element of the nucleotidyl transfer reaction and plays multiple roles in catalysis. When the MeA coordination is established, it aligns the reactive groups of 3'-OH(+1) and $\alpha$P(+2), and the active site proceeds to the $S_2$ reaction. The A-family polymerase is not able to coordinate MeA without properly oriented 3'-OH and $\alpha$P groups from two different nucleotides. This requirement satisfies two essential functions of nucleic acid polymerase, catalytic efficiency and fidelity of nucleotide selection, by using a single atom at the active site. The presence of MeA is essential for catalysis and thereby the MeA is a great sensor of Watson-Crick base pairing between DNA template and nucleotide although base pairings are 10–12 Å away from the catalytic site. Whereas the A-family polymerase uses the O-helix movement as an early checkpoint of nucleotide selection, this additional kinetic checkpoint right before the bond formation is able to greatly enhance the fidelity (10).

Due to highly conserved active site architecture, the reaction scheme of nucleotidyl transfer reaction of the N4 vRNAP should be universal to all A-family polymerase, including bacterial DNAP I (13, 14), bacteriophage DNAP (15), and also pol $\gamma$ in eukaryotes involved in the mitochondrial DNA replication (26). In contrast, the order of MeA and MeB bindings at the active site is different in multisubunit RNAP. The MeA is coordinated by three Asp residues in the absolutely conserved DFDGD motif in the largest subunit (27), and the MeB is coordinated at the active site without primer 3'-OH or nucleotide.
This difference is consistent with the fact that the A-family polymerase carries out only the nucleotidyl transfer reaction whereas the multisubunit RNAP is capable of not only nucleotidyl transfer but also RNA hydrolysis, which plays a role in the transcription proofreading (28).

In each TIC structure, distinct shapes and intensities of $F_o - F_e$ electron density maps corresponding to nucleotides and metals were revealed at their active sites (Fig. 2, b–e) although the times of soaking for each TIC preparation differ on the minutes time scale. Emerging evidence supports a hypothesis that the protein flexibility plays an important role in enhancing the enzyme catalysis speed (1, 29). In crystals, due to the crystal packing, protein flexibilities are restricted, therefore the nucleotidyl transfer reaction proceeds at a substantially slower rate (minutes) than in solution (milliseconds to seconds) (17, 21). Moreover, the RNAP-DNA complex is highly concentrated (5 mM) in small volume (0.3 nl) (“Experimental Procedures”). This unique environment allows diffusion of nucleotide plus metal to all binary complexes in short period of time and synchronizing the reaction of all molecules in crystal to reveal some hitherto undetected intermediates by freezing crystals sequentially and solving their high resolution crystal structures.

The mechanism of phosphoryl transfer reaction is explained by the two-metal-ion mechanism, which was proposed two decades ago (4, 5, 8). This model has been an important framework for explaining many reactions of nucleic acid enzymes, including DNAP, RNAP, self-splicing introns, and nucleases (6). Our study provided a new basis, chronology of substrate and metal bindings and protein conformational change, for the two-metal-ion mechanism. We demonstrated that the $X^A$ binds after $Me^B$ and nucleotides and does so immediately before the chemistry step, phosphodiester bond formation. The $X^A$ is a Lewis acid to reduce the $pK_a$ of the primer $3'\text{-OH}$ and aligns the reactive groups of $3'\text{-OH}$ and $\alpha$-phosphate to proceed through the $S_2$ reaction. The $X^A$ also satisfies the fidelity of nucleotide selection. The $X^A$ loading depends on presence of the primer $3'\text{-OH}$ group and other ligand positions (9, 30), and both reactive groups, $3'\text{-OH}$ and $\alpha$-phosphate, are involved in the $X^A$ coordination. Therefore, the $X^A$ can be a great sensor to monitor correct base pairing between DNA and nucleotide; once the Watson-Crick base pairs are established, the $X^A$ binds at the active site and completes the reaction. Our structures provide evidence that the $X^A$ binding is the final checkpoint of the nucleotide selection of A-family polymerase.

The proton transfer mechanism in the nucleotidyl transfer reaction has a longstanding question regarding the identification of a general base that accepts the proton to generate the $3'O$ nucleophile and a general acid, which provides a proton to stabilize the $PP_1$ leaving group. Although a conserved lysine residue at the $O$-helix has been proposed as a general acid in the reaction (31), K-670 in the N4 mini-vRNAP transcript initiation complex structures does not lie close to a bridging oxygen of the $\beta P$ of ATP(+2) (Fig. 4). However, the $-OH$ group of residue Y-612 is at a closer distance from the nonbridging oxygen at the $\beta P$ (+2) (Fig. 4). We thus ask the question if this Y-612 was lending its hydrogen to stabilize the negative charge developed on the $PP_1$ leaving group and serving the general acid in the reaction. A mutation Y571F at this position of T7 RNAP reduces its catalytic activity (32), which supports the proposed role of this conserved tyrosine as a general acid. In case of A-family DNAP, histidine instead of tyrosine is found at this position. A substitution of histidine (H932Y) reduced the specificity constant $k_{cat}/K_m$ of the human mitochondrial DNAP (pol $\gamma$) by $\sim$150-fold, and this effect is largely because of slowing down $PP_1$ release (33). Near neutral $pK_a$ of histidine imidazole group is a more efficient general acid, which might be a reason why DNAP is $>10$ times faster than RNAP for nucleotidyl transfer reaction. However, such a conclusion should await an ultra higher resolution structure or complementary studies capable of locating the proton between the Y-612 and the phosphate.

Time-resolved structure determination techniques have been developed to film biological processes at atomic or near-atomic resolutions (1–3). Nuclear magnetic resonance (NMR) spectroscopy has a clear advantage and is able to reveal the time scale of protein motions with atomic resolution (34, 35), but it is applicable to only well behaved smaller proteins and is not able to monitor the reaction over a time course. Kinetic x-ray crystallography, Laue diffraction, is able to deliver unprecedented detail of enzymatic reaction (2, 3, 36); however, it demands on ultrafast triggering of the reaction, largely restricting the application to light-induced reactions. Due to these obvious restrictions, complex reactions such as DNA replication and transcription have been excluded from the scope of time-resolved structural study. A simplistic time-resolved soak-trigger-freeze approach used in this study is able to trace the reaction pathway at ambient temperatures without any modification of enzyme and substrate and without any specific
equipment for x-ray data collection. The time-dependent soak-trigger-freeze x-ray crystallography can be applicable to other systems and become a general method to look directly at biological reactions (37).

Acknowledgments—We thank the staff at F1 of the MacCHESS for support crystallographic data collection; P. R. Carey and Y. Chen for discussions; and L. B. Rothman-Denes, P. C. Bevilacqua, S. J. Benkovic, and R. Yajima for critical reading of the manuscript. Figures were prepared using PyMOL.

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