

Binding of Mn-deoxyribonucleoside triphosphates to the active site of the DNA polymerase of bacteriophage T7

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Divalent metal ions are crucial as cofactors for a variety of intracellular enzymatic activities. Mg^{2+} , as an example, mediates binding of deoxyribonucleoside 5'-triphosphates followed by their hydrolysis in the active site of DNA polymerase. It is difficult to study the binding of Mg^{2+} to an active site because Mg^{2+} is spectroscopically silent and Mg^{2+} binds with low affinity to the active site of an enzyme. Therefore, we substituted Mg^{2+} with Mn^{2+} : Mn^{2+} that is not only visible spectroscopically but also provides full activity of the DNA polymerase of bacteriophage T7. In order to demonstrate that the majority of Mn^{2+} is bound to the enzyme, we have applied site-directed titration analysis of T7 DNA polymerase using X-ray near edge spectroscopy. Here we show how X-ray near edge spectroscopy can be used to distinguish between signal originating from Mn^{2+} that is free in solution and Mn^{2+} bound to the active site of T7 DNA polymerase. This method can be applied to other enzymes that use divalent metal ions as a cofactor. © 2011 International Centre for Diffraction Data. [DOI: 10.1154/1.3583156]

Key words: DNA polymerase, manganese, XAFS, metal cofactor, metalloenzyme

I. INTRODUCTION

The product of gene 5 of bacteriophage T7 is a DNA polymerase (gp5) that dissociates from the DNA after catalysis of only a few cycles of polymerization of nucleotides. Hence it is designated as a polymerase with low processivity. The processivity factor, *Escherichia coli* thioredoxin (trx), binds tightly (5 nM) (Huber *et al.*, 1986) in a one-to-one stoichiometry to a unique polypeptide loop (trx-binding domain) located in the thumb subdomain of gp5 (Bedford, Tabor and Richardson, 1997). The binding of trx converts gp5 to a highly processive polymerase that polymerizes hundreds of nucleotides before dissociating from the DNA template (Hamdan *et al.*, 2007; Tabor *et al.*, 1987). The utilization of nucleotides in the polymerization process is mediated by divalent metal ions. Mg^{2+} is abundant in the cell and is usually the divalent metal ion of choice in processes involving hydrolysis of nucleoside triphosphates. The crystal structure of gp5 in complex with trx, a primer-template, and a nucleoside 5'-triphosphate trapped in a polymerization mode is available (Figure 1). The active site of DNA polymerase binds two metal ions. One Mg^{2+} stabilizes the reactive state of the bound dNTP, and a second Mg^{2+} takes an active role in catalysis as postulated in the two metal ion mechanism (Steitz, 1998).

Mg^{2+} shields the negatively charged phosphate groups of the nucleotide triphosphate to avoid repulsion of the nucleophile during catalysis (Jencks, 1987). Mg^{2+} may also be involved in increasing the reactivity of the attacked phosphor by withdrawing electrons and stabilizing the leaving phosphate. Mg^{2+} also forms a bridge between the nucleophile (water) and the nucleoside triphosphate. Mg^{2+} may also induce structural rearrangements in the active site required for

precise positioning of the substrate. Probing Mg^{2+} using X-ray absorption spectroscopy (XAFS) is technically difficult (for review see Akabayov *et al.*, 2005). Therefore, since Mg^{2+} is spectroscopically silent, one approach to obtain information about the active site metal is its substitution by spectroscopically "visible" metal ions (Akabayov *et al.*, 2005). However, it is a challenge to study metal binding sites when the metal cofactors are weakly bound.

XAFS serves as a tool for directly monitoring the binding of Mn^{2+} to the active site of T7 DNA polymerase. XAFS provides high resolution structural and electronic information about a metal site by measuring the transition from core electronic states of the metal to excited electronic or continuum states. X-ray absorption near edge structure (XANES), spectral analysis near the electronic transition, is used to obtain information about the charge and the geom-

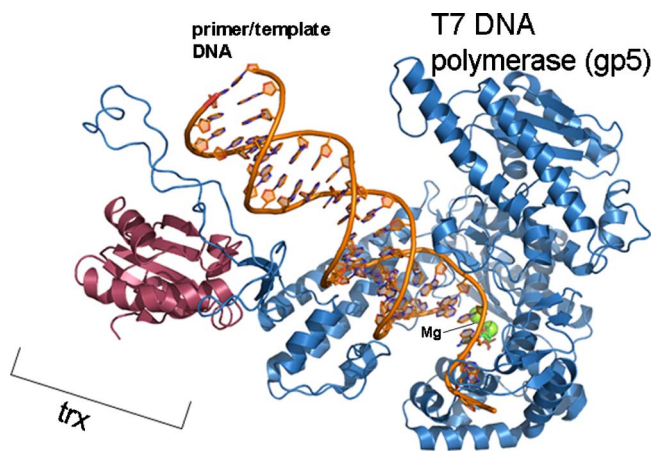


Figure 1. (Color online) Crystal structure of the bacteriophage T7 DNA polymerase (PDB entry 1t8e). The DNA polymerase (gp5) and the host encoded processivity factor trx are indicated. The figure was created using PyMOL (<http://www.pymol.org>).

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etry of the metal ion (Scott *et al.*, 1985). Previous studies on model compounds linked intensity patterns and shapes of XANES spectra to structural information on the metal binding site such as coordination number and geometry (Blumberg *et al.*, 1976; Shulman *et al.*, 1976; Westre *et al.*, 1997). Thus, XANES allows the study of the binding properties of a nucleoside triphosphate when Mn^{2+} is in the active site of T7 DNA polymerase. However, since binding of Mn to the polymerase active site is weak (approximately $20 \mu M$), the challenge was to verify that the signal originates from bound Mn^{2+} rather than from Mn^{2+} that is free in solution. Here we show that titration of T7 DNA polymerase to Mn-ddATP can be used as a fingerprint of the metal cofactor binding and allows the determination of the best conditions for probing the metal binding site by XAFS.

II. EXPERIMENTAL

A. Materials and reagents

Oligonucleotides were obtained from Integrated DNA Technology. All chemical reagents were of molecular biology grade (Sigma). Nucleotides were purchased from Roche Molecular Biochemicals. Radioactive materials were purchased from Perkin-Elmer. Wild-type and genetically modified gp5 were overproduced and purified using metal free buffers as described (Tabor and Richardson, 1989).

DNA polymerase activity was measured in a reaction containing 20 nM M13 ssDNA annealed to a 24-mer primer, 40 mM tris-HCl, pH 7.5, 2.75 mM $MgCl_2$ or $MnCl_2$, 10 mM DTT, 50 mM potassium glutamate, 0.25 mM dTTP, dGTP, dCTP, and $[\alpha\text{-}^{32}P]\text{-dTTP}$ (5 cpm/pmol), and 10 nM of T7 gp5/trx. The reaction was incubated at $37^\circ C$ for 10 min and terminated by the addition of EDTA to a final concentration of 20 mM. Aliquots of the reaction were spotted on DE-81 filters (Whatman), washed with ammonium formate, and radioactivity retained on the filters was measured.

B. Sample preparation for XAFS

The proteins were concentrated by centrifugation using Millipore Centricons (Bedford, MA) to yield a final concentration in the sub-mM range. The DNA used in each sample contained 21-mer primer 5'-CGAAACGACGGCCAGTGCCA-3' annealed to 26-mer template 5'-CCCCCTGGCACTGGCCGTCGTTTTTCG-3'. Each sample contained $300 \mu M$ $MnCl_2$ and dideoxy-ATP (ddATP) in a buffer of 40 mM Hepes, pH 7.5, 50 mM potassium glutamate, 1 mM DTT, 30% glycerol, and increasing amounts of T7 gp5 (32, 170, and $450 \mu M$) mixed with trx ($450 \mu M$) (see Figure 2).

Binding of Mn-dNTP to T7 DNA polymerase is a single step reaction of the type $A+B \rightleftharpoons AB$. The dissociation constant is given by

$$K_D = \frac{[A][B]}{[AB]},$$

where $[A]$ and $[B]$ are the concentrations of T7 DNA polymerase (B) and Mn-dNTP (A) and $[AB]$ the concentration of the complex in equilibrium.

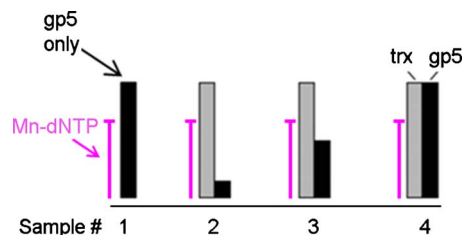


Figure 2. (Color online) Schematic representation of sample preparation. Bound nucleotide obtained upon titration of T7 DNA polymerase (gp5) above and below its binding constant to dATP ($20 \mu M$) using the binding equation (1). The four samples are depicted schematically in columns where black is the amount of DNA polymerase used, gray is the amount of trx, and the amount of Mn-ddATP is fixed as indicated ($300 \mu M$).

Expressing the equilibrium concentrations of the separate components in terms of initial concentrations (indicated by a subscript "0") and the concentration of the complex

$$A = [A_0] - [AB],$$

$$B = [B_0] - [AB].$$

We obtain

$$K_0 = \frac{([A_0] - [AB])([B_0] - [AB])}{[AB]}.$$

If we wish to have x fraction of A_0 in the complex AB :

$$[AB] = x[A_0]$$

then the following amount of B is required to have AB complex:

$$B = \frac{xK_D}{1-x} + xA_0, \quad (1)$$

A_0 , the concentration of Mn-ddATP, was kept constant at $300 \mu M$ for all samples. K_D , the dissociation constant, is $20 \mu M$ according to Patel *et al.*, 1991. Therefore, to obtain 90% binding of Mn ($x=0.9$), $300 \mu M$ Mn-ddATP (A_0) was mixed with $450 \mu M$ T7 DNA polymerase (B).

The components were mixed at $0^\circ C$ and immediately frozen in copper sample holders ($10 \times 5 \times 0.5 \text{ mm}^3$) covered with Mylar using liquid nitrogen.

C. XAFS data collection

XAFS data collection was performed at the National Synchrotron Light Source at Brookhaven National Laboratory, beam line X3B. The spectra were recorded as previously described (Akabayov *et al.*, 2009). Specifically, the Mn K -edge was measured in a fluorescence mode at cryogenic temperature (20 K). The beam energy was selected using a flat Si (III) crystal monochromator with an energy resolution (dE/E): $\sim 2 \times 10^{-4}$. The incident beam intensity was recorded using a transmission ion chamber and the fluorescence signal from the sample was measured using a 13-element germanium detector. For the calibration of the X-ray energy, the transmission signal of a Mn foil reference was measured simultaneously with the sample fluorescence. For each sample, several scans were collected to obtain a total of 1×10^6 counts. The beam position was varied for each scan to minimize radiation damage and samples were checked for

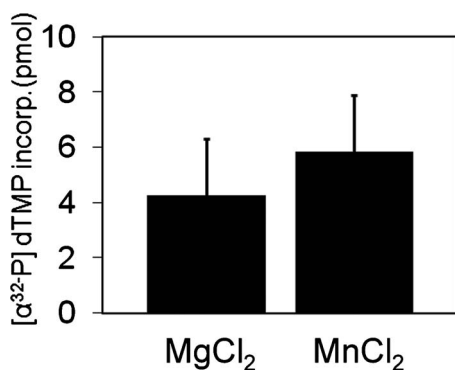


Figure 3. Effect of substitution of Mg with Mn on T7 DNA polymerase activity. DNA polymerase activity was measured in a reaction containing 40 mM tris-HCl, pH 7.5, 2.75 mM MgCl₂, 10 mM DTT, 50 mM potassium glutamate, 0.25 mM dTTP, dCTP, dGTP, and [α -³²P] dTTP, 20 nM primed M13 DNA, and 10 nM T7 gp5/trx. After incubation at 37 °C for 10 min, the amount of [α -³²P] dTMP incorporated into DNA was measured.

visual signs of photoreduction (burn marks) after each scan (30 min). Further protection of the sample from radiation damage was obtained by addition of DTT to the sample buffer serving as a free radical scavenger. In addition, the protein sample was exposed to X-ray under cryogenic temperature. Examination of the samples on SDS page after the exposure to the X-ray beam revealed no evidence of protein degradation caused by radiation damage (not shown).

D. XAFS data processing and analysis

The average Mn *K*-edge adsorption coefficient $\mu(E)$ was obtained after 4–5 independent XAFS measurements for each sample. To calibrate the X-ray energy, all spectra for each sample were aligned using the first inflection point of a reference Mn foil X-ray absorption spectrum (6539 eV). The measured intensities were converted to $\mu(E)$ and the smooth pre-edge function was subtracted from the spectra to get rid of any instrumental background and absorption from other atomic edges. The data [$\mu(E)$] were normalized and a smooth postedge background function was removed to approximate $\mu(E)$ in order to align all spectra. All XAFS data processing steps were performed using ATHENA software embedded in the IFEFFIT analysis package (Newville, 2001).

III. RESULTS AND DISCUSSION

Metal ions that serve as cofactors for enzymatic activities have a lower binding affinity compared to structurally bound metal ions. With a metal ion concentration of >300 μ M, which is necessary to obtain XAFS signal, a relatively high concentration of protein is required to ensure that majority of Mn²⁺ ions are bound and protein solubility is a limiting factor. Consequently, it is important to have a method that distinguishes bound metal ions from those that are free in solution. To study the binding properties of the active site of T7 DNA polymerase, we have substituted Mg²⁺ with Mn²⁺. Mn²⁺ is not only spectroscopically “visible” but Mn²⁺ can also bind to T7 DNA polymerase to yield a fully active enzyme (Figure 3). We used a site-directed titration

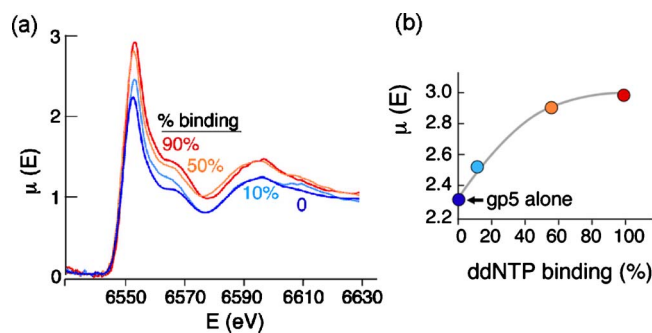


Figure 4. (Color online) (a) Site directed titration XANES analysis of T7 DNA polymerase above and below its binding constant to Mn-dNTP [according to Eq. (1), experimental]. Each sample contained the same concentration of Mn-dNTP (300 μ M) and increasing concentrations of T7 DNA polymerase (blue: 0 μ M; light blue: 32- μ M; orange; 170- μ M; red: 450- μ M) mixed with *E. coli* thioredoxin (450- μ M) (see Figure 2). (b) A typical binding curve when each point in this curve represents the maximum amplitude value of each XANES spectrum vs. dNTP binding [color coding as in (a)].

analysis with XAFS to demonstrate that the majority of the Mn²⁺ ions are bound to dNTP to form a stable protein-dNTP complex.

Binding of a chain terminating nucleotide dideoxy-ATP (ddATP) to the active site of gp5 was achieved by titration of gp5/trx above and below its binding constant to dNTP, $K_d = 20 \mu$ M (Patel *et al.*, 1991). The protein/nucleotide ratios were calculated based on the desired complex concentration and the equilibrium dissociation constant for the protein-ligand binding equilibria of the type: $A + B \rightleftharpoons AB$ (see Sec. II).

The XANES analysis enabled us to probe the coordination of the Mn-ddATP in the active site of gp5. The evolution of the amplitude of the X-ray absorption coefficient upon Mn-ddATP binding to T7 DNA polymerase as a function of the enzyme concentration shows that in the absence of trx (and with higher amounts of gp5), there is a low $\mu(E)$ of the near edge spectrum (Figure 4, blue, gp5 alone). The signal of ddATP binding to the active site of gp5 evolved when increasing amounts of gp5 were added to a fixed amount of trx (Figure 4, cyan, orange, and red circles, respectively).

Probing metal binding sites of enzymes by XAFS is one approach to explain the changes that occur in critical metal centers during catalysis. In XAFS, the metal is the probe for the detection of sensitive changes in its local structure as well as its intrinsic chemical properties. Spectral analysis near the electronic transition, XANES, provides information on the charge state and the geometry of the metal (Scott *et al.*, 1985). Studies on model compounds showed that pre-edge shapes and intensity patterns can be correlated with coordination number and geometry (Blumberg *et al.*, 1976; Shulman *et al.*, 1976; Westre *et al.*, 1997). We titrated T7 DNA polymerase at concentrations above and below the binding constant to Mn-ddATP. Binding of Mn-ddATP to T7 DNA polymerase results in an increase of the amplitude of the corresponding XANES signal as presented in Figure 4(a). The change in amplitude of the XANES highest peak with increasing protein concentration closely resembles a typical binding curve [Figure 4(b)]. This titration analysis allowed us to probe the conditions necessary for retaining the complex ddATP bound to the active site of T7 DNA polymerase

through Mn^{2+} . This method should be applicable to any protein binding metal ions in the high μM range. Such analysis is crucial when measuring the binding properties of the active site of an enzyme with low binding affinity to metal ions.

IV. CONCLUSION

Our results demonstrate a unique application of XAFS for characterizing the binding properties of metal ions to proteins especially when the binding affinity of the protein to the metal ion is low. The titration analysis presented here allows the differentiation of signal originating from bound or free metal ions and, subsequently, determination of the best sample conditions for the study of metal ions in the binding site. This application simplifies data analysis and eliminates the need for data manipulations such as principal component analysis.

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