The Role of Zinc and the Reactivity of Cysteines in *Escherichia coli* Primase[†]

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ABSTRACT: Primase is the zinc metalloenzyme responsible for synthesizing RNA primers for use during DNA synthesis. To establish whether the zinc played a catalytic or structural role, the zinc was removed and the activity of the apoprimase determined. The zinc was removed with *p*-(hydroxymercuri)-benzenesulfonate (PMPS), which covalently reacts with cysteine sulfhydryls, EDTA was added to chelate the zinc, DTT was added to remove the PMPS from the apoprimase, and then the apoprimase was separated from the small molecules. The resulting apoprimase was fully active, indicating that the zinc played a structural role but not one involved in thermodynamic folding/unfolding. PMPS and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) cysteine reactivities indicated that the cysteines fell into three categories: one or two were fast-reacting, three were zinc-ligating, and two or three were slow or nonreacting. The major distinction between apoprimase and natural primase was that apoprimase became inactivated during storage at 4 °C for 10 days. Storage-induced inactivation correlated with disulfide bond formation and could be reversed by incubation with a mild reducing agent. Apoprimase oxidation also prevented zinc reconstitution which was only achieved with freshly-reduced enzyme, indicating that the zinc-ligating cysteines participated in the inactivating disulfide bonds. The conclusion was that, in natural primase, the zinc prevented disulfide bond formation which, in turn, prevented inactivation. The zinc reconstitution studies identified a strong and a weak zinc binding site. Zinc could be prevented from binding to the weak site by the presence of magnesium, indicating that the weak site may be the catalytic magnesium site in which two of the seven cysteines were located.

Primase is a zinc enzyme (Ilyina et al., 1992; Mendelman et al., 1993, 1994; Stamford et al., 1992) that is a ssDNA-dependent[1] RNA polymerase. As an RNA polymerase, it catalyzes two types of reactions: nucleic acid polymer initiation and elongation. Zinc appears to be important for the function of most RNA polymerases since there is only one subfamily that lacks it, the bacteriophage T-series transcription RNA polymerases. Given that the zinc is found in most RNA polymerases but not DNA polymerases, it is likely that it is somehow involved in polymer initiation since that function is specific to RNA polymerases. As for every metalloprotein, there are two possible roles for the metal: catalytic or structural. One obvious potential catalytic role in primase would be that the zinc acts as a Lewis acid to react with water to create the hydroxyl used to deprotonate the 3′-hydroxyl on the reacting ribose. Several potential structural roles for the zinc in primase include global folding stability, protein−protein interactions, and DNA sequence-specific binding. In fact, excellent evidence has been presented that the role of the zinc in bacteriophage T7 primase/helicase has to do with primer intiation sequence specificity (Bernstein & Richardson, 1988; Mendelman et al., 1994). The actual role of zinc in this case must be structural in that it allows a portion of the primase backbone to fold into a particular structure, thereby exposing the critical hydrogen bonding residues that would result in sequence specificity. Sequence-specific initiation could be achieved by binding a specific ssDNA sequence, by binding the first two initiating nucleotides, or by creating quasi-duplex DNA by binding all three.

The biological role of primase is to synthesize short RNA polymers called "primers" during DNA replication. An RNA polymerase is required to carry out this function because they can initiate polymers whereas a DNA polymerase cannot. Primase is used rather than the transcription RNA polymerase because it synthesizes RNA polymers specifically at the replication fork. After the duplex DNA has been separated into two single strands by the action of helicases, primase initiates polymer synthesis once on each leading strand ssDNA and repeatedly on the lagging strand ssDNA (Kornberg & Baker, 1992; Marians, 1992; McHenry, 1991; McMacken et al., 1987). The RNA/DNA heteropolymers created on the lagging strand, called Okazaki fragments, are an average 1500 nucleotides in length (Okazaki et al., 1968; Wu et al., 1992) and begin with an 11 ± 1-ribonucleotide primer (Kitani et al., 1985; Zechner et al., 1992). After one Okazaki fragment is complete, other replication enzymes remove the short RNA polymers, fill in the gap with DNA, and then ligate the Okazaki fragment to create the completed high molecular weight lagging strand.

Primase has low affinity and low catalytic activity on ssDNA templates (Swart & Griep, 1995) and is inactive on ssDNA coated with SSB. These features prevent primase from synthesizing RNA polymers at random along the lagging strand ssDNA template. DnaB helicase stimulates primase activity many-fold (Arai & Kornberg, 1981), and DnaB helicase is limited to action at the replication fork

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ICP-MS, inductively coupled plasma-mass spectrometry; PAR, 4-(2-pyridylazo)-resorcinol; PMPS, *p*-(hydroxymercuri)benzenesulfonic acid; SSB, *E. coli* ssDNA binding protein; ssDNA, single-stranded DNA.

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Table 1: Primase Purification from an Overproducing Strain

fraction	total activity ($\times 10^6$ units)	total protein[a] (mg)	yield (%)	specific activity (units/mg)
(I) lysate supernatant	800	30000	(100)	27
(II) ammonium sulfate	470	1305	59	360
(III) heparin-agarose	440	1043	55	420
(IV) Q-Sepharose	260	188	33	1380

[a] Total protein in fractions I and II was quantitated with Coomassie Blue using immunoglobulin G as a standard. Total protein in fractions III and IV was quantitated according to primase's extinction coefficient of 0.73 $(mg/mL)^{-1}$ cm^{-1}. The Coomassie assay provides a reasonable estimate of bulk protein but underdetermined pure primase. Because of this, the total protein did not appear to decrease much between fractions II and III even though the amount of protein was reduced by half during this step.

(LeBowitz & McMacken, 1986). Thus, helicase binding specificity stimulates primer synthesis to occur at the replication fork.

Primase initiates RNA polymer synthesis at the sequence d(CTG) within a ssDNA template (Swart & Griep, 1993; Yoda & Okazaki, 1991; Yoda et al., 1988). Initiation begins with ATP and GTP complementary to the thymine and cytosine and proceeds in the usual direction. The lengths of primers are rarely shorter than 9−12 nucleotides. Replacement of the initiation sequence by d(TTG) results in a template that cannot direct primer synthesis (Swart & Griep, 1993). This establishes that primase is less specific *in vivo* since ∼44% of primers begin pppApG and ∼13% pppApA (Yoda et al., 1988). The lower initiation sequence specificity of primase *in vivo* has been hypothesized to result from its interaction with DnaB helicase (Yoda & Okazaki, 1991; Yoda et al., 1988).

Removal of a metal from a metalloprotein is the first step to establish whether it plays a catalytic or structural role. If the zinc plays a catalytic role, then its removal should result in an inactive apoenzyme. If the role is structural, its removal may or may not alter activity depending on the extent of its importance for proper protein folding. After removal of the zinc, primase was active and could be reconstituted with zinc only when kept in a reduced state, indicating that the role of the zinc had been to prevent inhibitory disulfide bond formation.

EXPERIMENTAL PROCEDURES

Buffers. Buffer A is 50 mM MOPS, pH 7.0, 5% glycerol, 5 mM DTT, and 1 mM EDTA. Buffer B is 50 mM Tris, pH 7.4, 5% glycerol, and 5 mM DTT. HKg buffer is 50 mM HEPES, pH 7.5, 100 mM potassium glutamate. HKgD buffer is HKg buffer plus 5 mM DTT. TE buffer is 10 mM Tris, pH 8.0, 0.1 mM EDTA. All buffers used in the zinc removal and apoprimase activity assays were passed through a Chelex 100 (Bio-Rad, Richmond, CA) column to remove potentially contaminating metals. In addition, gloves were worn, and metal-free micropipet tips were used.

Primase Purification. Primase isolation was modified from a previous procedure (Swart & Griep, 1993) and is presented here in greater detail (Table 1). The primase-overproducing plasmid pRLM96 in the *E. coli* host RLM569 was kindly provided by Dr. Roger McMacken (Johns Hopkins University). On this plasmid, the primase gene was under the control of the bacteriophage λ P_L promoter, and the vector also contained an ampicillin-resistance gene. The expression of the λ P_L promoter is controlled by the temperature-sensitive λ cI857 repressor which represses at 30 °C and derepresses at 42 °C (Remaut et al., 1981). Large-scale growth of cells was performed by the University of Colorado Cancer Center (Denver) in a 200-L fermentator. When cell growth at 37 °C had reached an OD_{600} of 1.0, the temperature of the culture was increased to 46 °C over 10 min and then returned to 37 °C over 15 min. The culture was allowed to grow for an additional 2 h (final OD_{600} of 1.6). The cells were harvested, lysed, and centrifuged to remove lysed cells according to standard protocols (McHenry & Kornberg, 1977). The protocol described below uses 300 g of cells from the fermentation run, and all procedures were performed at 4 °C. Protein in the lysis supernatant was precipitated by the addition of 0.30 g of ammonium sulfate for every milliliter of solution. The solution was stirred for 30 min and then centrifuged at 22000*g* for 30 min. The pellet was resuspended in 125 mL of 50 mM Tris, pH 7.5, 20% sucrose, 5 mM DTT, and 250 mg/mL ammonium sulfate and centrifuged as previously described. The pellet was resuspended in 100 mL of buffer A containing 50 mM NaCl and dialyzed overnight versus buffer A with 50 mM NaCl.

The dialyzed sample was loaded on a heparin−agarose column (2.5 × 16 cm) (Bio-Rad Affi-Gel heparin) and washed with 250 mL of buffer A containing 50 mM NaCl. The bound proteins were eluted using a 400-mL gradient from 50 to 600 mM NaCl with a flow rate of 1.33 mL/min. Primase eluted at the leading edge of the first peak (at about 100 mM NaCl). The primase-containing fractions were loaded onto a Q-Sepharose column (2.5 × 16 cm) (Pharmacia) and washed with 250 mL of buffer B containing 50 mM NaCl. Primase eluted at about 200 mM NaCl in a 400-mL 50−250 mM NaCl gradient at a flow rate of 1.33 mL/min. The leading edge of the primase-containing fractions coeluted with a deoxyribonucleotide-specific 3′→5′ exonuclease (Swart and Griep, unpublished observation). The exonuclease was easily avoided by pooling only fractions containing more than 50% of the peak fraction activity. Fractions were analyzed for purity by 7% SDS−polyacrylamide gel electrophoresis followed by either Coomassie Brilliant Blue staining (Laemmli, 1970) or silver staining (Merril et al., 1980). Analysis by SDS−polyacrylamide gel electrophoresis revealed that the final protein was greater than 99% pure. On occasion, low molecular weight impurities have been observed in a preparation which strongly resembles primase-limited proteolytic digestion products. Once these impurities are present in a given preparation, it is difficult to remove them chromatographically. The best way to avoid them is to isolate primase as rapidly as possible. Primase concentration was determined using its extinction coeffcient of 47 800 M^{-1} cm^{-1} (0.73 mL mg^{-1} cm^{-1}) at 280 nm (Swart & Griep, 1993). With this procedure, it was possible to purify 188 mg of primase from 300 g of cell paste. After rapid freezing in liquid nitrogen and storage at −70 °C, primase was stable for over 3 years.

A primase standard curve was determined in the coupled RNA/DNA synthesis assay for various fractions in a Q-Sepharose chromatographic peak, and it was found that the assay was nonlinear with respect to primase concentration (Figure 1). This nonlinearity occurs because primase carries out the rate-limiting function and the signal is generated by

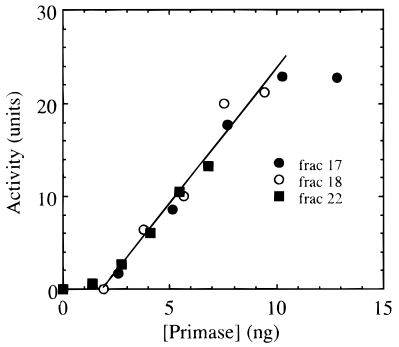

FIGURE 1: Primase standard activity in the coupled RNA/DNA assay. The activities of three fractions from a Q-Sepharose elution are shown. The amount of primase was determined from the absorbance using its extinction coefficient.

DNA polymerase III holoenzyme which elongates from the slowly synthesized RNA primer (Griep & McHenry, 1989). When the primase specific activity is calculated from the slope of the velocity versus enzyme concentration plot (Figure 1), it is greater than that observed during purification (compare Tables 1 and 2).

Other Proteins. Holoenzyme [fraction IV, 300 000 units/mg (Maki et al., 1988)] and SSB (Lohman et al., 1986; Swart & Griep, 1993) were isolated using previously published procedures.

Nucleic Acids. M13Gori ssDNA, a chimeric ssDNA consisting of a 2216-nucleotide G4 complementary strand origin inserted into a 6407-nucleotide M13 ssDNA (Kaguni & Ray, 1979), was isolated according to the following protocol. One liter of JM109 *E. coli* was grown in LB + 1 mM thiamin media until the OD_{600} was 0.5 (corresponding to 1×10^7 cells/mL), infected with with M13Gori bacteriophage (a gift from Dr. Charles S. McHenry, University of Colorado Health Sciences Center, Denver) at a multiplicity of infection of 100, and grown overnight. The cell and phage suspension was centrifuged at 8300*g* for 30 min at 4 °C to remove the cells. Poly(ethylene glycol) 8000 (Sigma, St. Louis) at a final 5% concentration was added to the resulting phage suspension, briefly stirred, and then centrifuged at 15300*g* for 30 min at 4 °C. The phage precipitate was washed by resuspending in 20 mL of TE buffer followed by centrifugation. Poly(ethylene glycol) 8000 (final 5%) and 500 mM NaCl were stirred into the resulting supernatant, and then the solution was centrifuged at 15000*g* for 30 min at 4 °C. The phage precipitate was washed by resuspending in 5 mL of TE buffer, centrifuging, and keeping the supernatant. The final phage preparation had an A_{260}/A_{280} ratio of 1.08 and an A_{260}/A_{340} ratio of 26.

The concentrated phage were SDS-denatured and subjected to two Bio-Rad A-5m gel filtration columns, the first one equilibrated with TE buffer + 0.5% SDS to separate viral coat proteins from the viral ssDNA and the second one equilibrated with TE buffer to remove the SDS from the viral ssDNA. SDS (final 0.5%) was added to the phage preparation, the solution heated for 15 min at 65 °C, KCl added to final 500 mM, and the sample cooled on ice for 10 min. The solution was centrifuged at 16000*g* for 30 min at 4 °C to pellet the potassium−SDS−protein aggregate. The supernatant was loaded on an A-5m column (greater than 10 times the volume of the sample) that was equilibrated with TE buffer + 0.5% SDS. The ssDNA eluted in the void volume and had an A_{260}/A_{280} ratio of 1.84. The fractions were collected, and the ssDNA was concentrated by adding NaCl (final 200 mM) and 2-propanol (final 50%) to the fractions which were then cooled for 20 min at −70 °C. The samples were centrifuged at 16000*g* for 30 min at 4 °C to pellet the salty ssDNA. The ssDNA was resuspended in TE buffer, loaded, and then eluted from the A-5 m column equilibrated with TE buffer. The final ssDNA preparation had an A_{260}/A_{280} ratio ranging from 1.84 to 1.86 and an A_{260}/A_{340} ratio ranging from 500 to 2000. The final preparation migrated as a single species in 0.55% agarose (SeaKem LE agarose from FMC, Rockland, ME) in Tris−borate−EDTA buffer.

Coupled RNA/DNA Synthesis. One unit of primase (or holoenzyme) was defined as the amount needed to incorporate 1 pmol of (total) deoxyribonucleotide/min into acid-precipitable DNA during a 5-min incubation with M13Gori ssDNA at 30 °C. The assay was performed as previously described (Griep & McHenry, 1989; Johanson & McHenry, 1980) except that the Standard Assay Buffer was 50 mM HEPES, pH 7.5, 100 mM potassium glutamate, and 10 mM DTT. All values have been adjusted for the percent retention of the replicated DNA on the GF/C filters (Griep & McHenry, 1989) which was 80%.

PMPS/PAR Assay. The moles of sulfhydryls reacted with PMPS was determined spectrophotometrically at 250 nm (Boyer, 1954; Giedroc et al., 1986; Hunt et al., 1984). The difference extinction coefficient for the PMPS−sulfhydryl charge-transfer complex was determined to be 3.00 mM^{-1} cm^{-1} at 250 nm in HKg buffer by titrating a fresh PMPS solution into a freshly diluted 2-mercaptoethanol solution. The moles of zinc released to the solution was determined spectrophotometrically at 500 nm as had been done by others (Giedroc et al., 1986; Hunt et al., 1984). Since the difference extinction coefficient for the $PAR_2H_{0-2}Zn$ complex is pH-dependent (Tanaka et al., 1968), its value in our buffer was determined. By titrating zinc acetate stock solution (up to 30 μM) into a fresh PAR solution (100 μM), the $\Delta\epsilon$ values at 500 and 530 nm were determined to be 82.2 and 46.4 mM^{-1} cm^{-1}, respectively. Typically, the zinc content of 10 μM primase was determined in these assays.

Apoprimase was created by incubating primase with 5 or more equiv of PMPS in the presence of 2 mM EDTA for 10 or more min, incubating with 5 mM fresh DTT for more than 10 min to release the cysteine-bound PMPS, and then either S-200 gel filtration or multiple Centricon-10 washes. If necessary, the enzyme was concentrated and its buffer exchanged using a Centricon-10 apparatus (Amicon, Inc., Beverly, MA). Prior to use, the apparatus was centrifuged with a solution containing either EDTA or 1,10-phenanthroline and then 4 or more times with Chelex-treated HKg buffer to ensure no metal contamination. The phenanthroline was advantageous in this regard because the absorbance of the filtrate could be monitored to determine how much remained in the retentate. After the crude apoprimase mixture was centrifuged for the first time, HKgD buffer was added and the sample centrifuged again. This was called the first wash. The sample was then washed 4 times with HKg buffer to remove any residual DTT. The A_{280}/A_{250} ratio for primase and apoprimase was between 2.00 and 2.25, and the wavelength of the absorption trough was 250.5 ± 1 nm. These values were used to establish that PMPS removal had been complete because PMPS absorbs at 250 nm.

Table 2: Zinc Content and Activity of Primase and Apoprimase[a]

primase	zinc/primase PMPS/PAR	zinc/primase ICP-MS	total metals ICP-MS	specific activity (units/ng)
primase	1.07 ± 0.02	0.79 ± 0.09	0.77 Zn	2.6
apoprimase	0.01, 0.01	ND	ND	2.4

[a] Primase zinc content was determined by the PMPS/PAR assay and quantitative ICP-MS. All values are for more than 3 samples. Total primase metal content was determined by ICP-MS in the semiquantitative mode, and the value was from the average of two samples. The only metal ion present at higher than 20 ppb that correlated with primase concentration was zinc. The specific activity of apoprimase was determined on freshly reduced samples in the radionucleotide incorporation assay. ND means not determined.

ICP-MS Quantitation of Zinc. Samples were measured for either total metal content or zinc alone by Karl Baumgarten at the University of Nebraska−Lincoln ICP-MS Center using a Perkin Elmer instrument. Total metal content was determined in the semiquantitative mode, and accurate zinc concentration was determined using the quantitative mode. Prior to analysis in the semiquantitative mode, 1 mL of sample was spiked with 50 ppb of indium and diluted into 1% HNO_3 (10 mL final). Detector response across the spectrum of masses was scaled to the indium spike intensity (50 ppb). To control for varying detector response at widely divergent masses, a standard containing 50 ppb each of five different metals was prerun, and the appropriate analytical adjustments were made.

Prior to analysis in the quantitative mode, the detector response was scaled to a set of zinc acetate standards. The five runs of five standard solutions (0, 50, 100, and 200 ppb of heat-dried zinc acetate in 1% HNO_3, respectively) yielded values of 6.6 ± 2.0, 49.4 ± 0.4, 105.4 ± 2.8, and 214.1 ± 5.4 ppb. Besides scaling the instrument, these values indicated that the technique was more accurate than it was precise. Samples were diluted to 10 mL and contained. The final primase (and zinc) concentration was in the range of 5−200 ppb (=ng of metal/mL).

RESULTS

Primase Metal Content. The total metal content was determined by ICP-MS in its semiquantitative mode. The only di- or trivalent metal in our primase preparation was zinc (Table 2) which was found to be present in the ratio of 0.79 zinc/primase. The other elements present in significant amounts were selenium (40 ± 7 ppb) and iron (470 ± 70 ppb). The concentrations of selenium and iron in the samples did not correlate with a 2-fold change in primase concentration, indicating that they were not bound to the primase. These two elements were present at elevated concentrations in the DTT-containing primase storage buffer and, even though the buffer contribution has been corrected for, are likely to be correlated to the DTT. When the zinc concentration was determined with the instrument in its quantitative mode, the ratio of zinc/primase was 0.77 (Table 2). This value reflected the total content of zinc per primase.

Removal of the Zinc and Study of the Cysteine Reactivity of Primase. It was not possible to remove the zinc from primase by dialysis against an EDTA solution for 3 days at 4 °C. This indicated that primase has an affinity for zinc greater than does EDTA and that a different approach was required for removing the zinc. Based on amino acid sequence similarity, the zinc in primase has been proposed

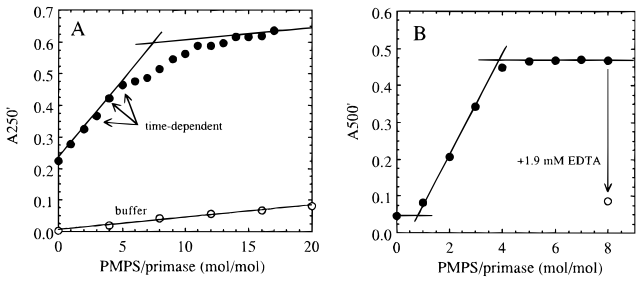

FIGURE 2: Primase cysteine reactivity and zinc release. (A) The change in absorbance at 250 nm was monitored while equivalents of PMPS were added to 10 μM primase in HKg buffer. (B) The change in absorbance at 500 nm was monitored while equivalents of PMPS were added to 10 μM primase in HKg buffer containing 50 μM PAR. Each value was recorded 2 min after adding PMPS. The absorbance values have been adjusted for dilution effects.

to be bound by three cysteines and one histidine (Ilyina et al., 1992). One approach to displacing sulfhydryl-bound zinc from a protein is to add equivalents of an organomercurial such as PMPS (Hunt et al., 1984). When PMPS was titrated into a 10 μM primase solution, the absorbance at 250 nm increased due to the formation of mercury−thiolate charge-transfer complexes (Figure 2A). The first 4−5 equiv of PMPS reacted rapidly with primase to give the absorbance increase shown. At 5 equiv of PMPS, primase became less soluble. The remaining cysteines did not form mercury−thiolate bonds stoichiometrically. The poor reactivity indicated that the three remaining cysteines were not readily accessible to the reagent. At high enough PMPS, however, all of the cysteine did bind as indicated by the appropriate incremental increase in ΔA.

Next, the PMPS titration was performed in the presence of the zinc binding dye PAR (Figure 2B). In this titration, the formation of the zinc−PAR_2 complex was measured by its absorbance at 500 nm. The first PMPS equivalent did not result in a significant absorbance increase, indicating that little zinc was released. The next 3 PMPS equiv liberated 1.07 zinc/primase (Table 2). This amount of PMPS-released zinc was similar to that determined by ICP-MS and indicated that all of the bound zinc was released.

Preparation and Activity of Apoprimase. Apoprimase was prepared by reacting it for 10 min with 5 equiv of PMPS in the presence of 2 mM EDTA and then adding DTT to displace the primase-bound PMPS. The apoprimase was transferred to fresh HKg buffer by several washes in a Centricon-10 concentration apparatus. When, instead of the concentration apparatus, the solution was applied to a S-200 gel filtration column, the apoprimase eluted from the gel filtration column at the same location as monomeric primase, indicating that the apoprimase was not significantly aggregated. The zinc content of apoprimase prepared by this method was negligible (Table 2).

When freshly prepared, apoprimase was fully active in the coupled primer synthesis/DNA synthesis assay with a specific activity of 2.4 units/ng. A trivial explanation for this activity would be zinc reconstitution *in situ*. However, all of the solutions except those containing the DNA polymerase III holoenzyme, SSB, and magnesium acetate had been treated to remove metals prior to use in the assay (see Experimental Procedures). Also, in an attempt to foil potential metal contamination, 100 μM EDTA was added to the reaction mixture prior to apoprimase.

A clue as to one of the roles of the zinc was obtained in activity assays of the apoprimase following storage. When apoprimase was stored at 4 °C for 3 days, its specific activity was 0.2 unit/ng and by 10 days was negligible. Full activity could be recovered by treating the enzyme with 10 mM DTT for 5 min prior to assaying (2.3 units/ng). In contrast, primase stored at 4 °C for 10 days does not lose activity. Because of the many steps taken to prevent zinc or other metal contamination in all of the buffers except the holoenzyme, SSB, and magnesium solutions, we discounted the possibility that the reduced apoprimase was undergoing *in situ* reconstitution. Despite these concerns, it was clear that stored apoprimase was inactive and the presence of zinc in native primase prevented the inhibition.

To prove that *in situ* zinc reconstitution was not occurring because of the holoenzyme, SSB, or their storage buffers, we tested the activity of primase and apoprimase in a simple primer synthesis assay that does not require the addition of either of these proteins (Swart & Griep, 1993). We have found that primase will synthesize RNA polymers on a ssDNA template with the sequence d(CAGACACACACA-CACTGCAAAGC) because it contained several features: the d(CTG) primer initiation sequence; the minimum 6 nucleotides flanking the d(CTG) on the 3′-side (Swart & Griep, 1993); 14 nucleotides 5′ to the d(CTG) so that the longest template-dependent primer should be 16 nucleotides if it begins complementary to the unique thymine in the template. The single guanine was present in the template for use in other studies not related to the present one (Swart & Griep, 1995). In the experiment, [α-^{32}P]UTP was used as the label so that all RNA polymers could be visualized regardless as to whether they initiated complementary to the thymine. Analysis revealed that primase and freshly prepared apoprimase had similar activities while that of 1-day-stored apoprimase was greatly reduced (Figure 3). The RNA primers that were 16 nucleotides and shorter had sequences that were complementary to the template, indicating that initiation specificity had not been lost when the zinc was removed. In addition, RNA polymers that were longer than the template, called "overlong primers", whose origin has not been fully elaborated yet, did not correlate with the presence or absence of zinc. All features of primer synthesis observed with native primase were observed with freshly prepared apoprimase but not with stored apoprimase. These results reflected those obtained in the coupled RNA/DNA synthesis assay with holoenzyme present and confirmed that there was little likelihood for *in situ* primase reconstitution from contaminating zinc.

Apoprimase Reconstitution with Zinc. Zinc reconstitution was monitored by either of two methods, intrinsic fluorescence quenching or PMPS/PAR quantitation (Table 3). For both methods, the issue of stored versus freshly prepared apoprimase was important. Stored apoprimase could be reconstituted only if DTT was present or if it had been first incubated with DTT and then the DTT was removed. On the other hand, stored apoprimase not treated like this would not reconstitute with zinc even after extensive dialysis or incubation.

It was possible to monitor zinc reconstitution by fluorescence. Even though the primase intrinsic fluorescence was not sensitive to the addition of magnesium, ATP, or ssDNA, we found that the intrinsic fluorescence of reduced apoprimase was sensitive to transition metal reconstitution. In the

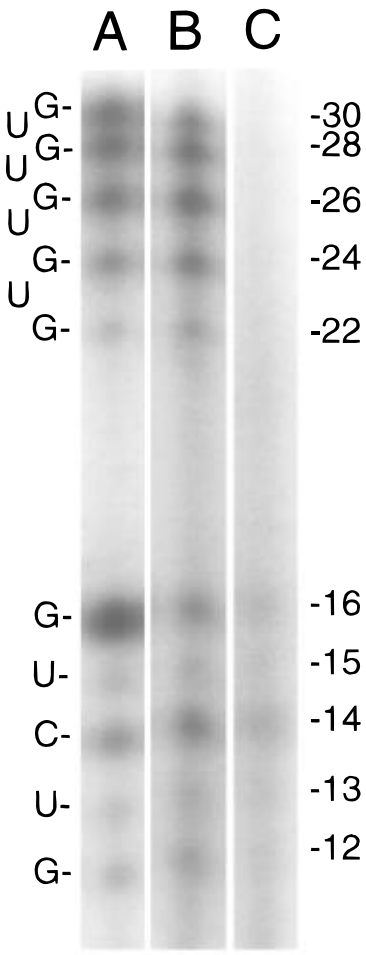

FIGURE 3: Primer synthesis activity of primase and apoprimase. Primase (lane A), apoprimase that had been treated with DTT and then had the DTT removed (lane B), and 1-day-stored apoprimase (lane C) were tested for their ability to synthesize RNA primers complementary to the ssDNAtemplate, 5′-CAGACACACACA-CACTGCAAAGC. Each enzyme was assayed in triplicate, but only a representative lane from each is shown here. The sequence of the RNA polymer is shown along the left side of the image and their lengths along the right.

presence of DTT, zinc addition caused proportional quenching of apoprimase fluorescence until almost 2 (1.72 $\pm$ 0.08) zinc equiv had been added (Table 3). The metal equivalents were in excess of that originally bound to primase. Adding 1 mM EDTA did not cause a significant reversal of the fluorescence quenching. Since it was possible that the metals were binding to "magnesium" binding site(s) in addition to the "zinc finger" site, the titrations were performed in the presence of 10 mM magnesium acetate. Under these conditions, only 1 (1.02 $\pm$ 0.02) zinc equiv was observed to be reconstituted, indicating that magnesium prevented the second zinc equivalent from binding. The extent of fluorescence quenching decreased only 2% when the titration was performed in the presence of magnesium, suggesting that the "magnesium" binding site was not near a fluorescence sensitive residue.

When reconstituted primase was prepared by incubation with a 5-fold molar zinc excess followed by multiple washes in a centrifugal concentrator, only 1 zinc equiv was bound tightly (Table 3). This was the same amount bound to native primase that was treated similarly. Washing different preparations of reconstituted primase with either EDTA or magnesium did not alter the ratio of zinc to primase. The

Table 3: Zinc Reconstitution of Apoprimase

fluorescence quenching[a]	reconstitution buffer	zinc/primase	% quenching
titrate zinc	HKgD	1.72 ± 0.08	14 ± 2
titrate zinc	HKgD + 10 mM $MgOAc_2$	1.02 ± 0.02	12 ± 2

incubation/ washing[b]	first wash buffer	zinc/primase
native (5 trials)	HKg	0.91 ± 0.11
trial 1 (9 trials)	HKg	1.06 ± 0.26
trial 2 (6 trials)	HKg + 10 or 100 μM EDTA	0.92 ± 0.31
trial 3 (3 trials)	HKg + 1 or 10 mM $MgOAc_2$	1.15 ± 0.13

[a] In the fluorescence quenching method, apoprimase (10 μM) at 30 °C in the indicated buffer was excited at 280 nm and fluorescence emission monitored at 340 nm. Aliquots of zinc chloride were added and the fluorescence intensity recorded. The values for zinc/primase and % quenching were taken from the point at which the added zinc caused no additional fluorescence change. Each value and its standard deviation were determined from three titrations. [b] In the incubation/ washing method, apoprimase (10 μM) at 4 °C in HKgD + 50 μM $ZnAc_2$ was incubated for 5 min, diluted 2-fold with HKg, concentrated in a centrifugal concentrator, washed with the first wash buffer, and then washed an additional 4 or more times with HKg. The zinc in the reconstituted primase was quantitated in the PMPS/PAR assay. The number of trials from which the average and standard deviation were determined are indicated.

Table 4: Cysteine Reactivity of Primase and Apoprimase[a]

	reactive cysteine/primase (mol/mol)	
primase species	native	denatured
primase	2.0 ± 0.6	6.9 ± 0.4
apoprimase, stored	1.2 ± 0.2	3.5 ± 1.2
apoprimase, DTT-reduced	4.7 ± 1.3	4.9 ± 2.0
reconstituted primase	1.8 ± 0.1	7.2 ± 0.1

[a] DTNB reduction (cysteine reactivity) of native primase was measured in HKg buffer using an extinction coefficient of 15 300 M^{-1} cm^{-1} and denatured primase in 7 M guanidinium chloride using 13 000 M^{-1} cm^{-1}. Oxidized apoprimase was apoprimase that had been stored at 4 °C in the absence of a reducing agent. DTT-reduced apoprimase was oxidized apoprimase that had been incubated with 5 mM DTT for at least 5 min prior to buffer exchange into degassed HKg using a Centricon 10 apparatus.

more weakly bound zinc that had been observed in the fluorescence quenching trials was apparently removed by dilution during the numerous washings. The conclusion from the two reconstitution experiments was the same—one reconstituted zinc was strongly bound to primase.

Cysteine Availability of Primase and Apoprimase. DTNB is a chromogenic reagent for reactive sulfhyrdryls (Habeeb, 1972). We used it to establish the availability of cysteines in freshly prepared versus stored apoprimase and to compare those to native and reconstituted primase (Table 4). In nearly all of the conditions and species of primase tested, the reaction of the cysteines with DTNB was complete by 30 s. The exceptions to this were the native conformations of primase and reconstituted primase in which one cysteine reacted within 30 s and the second cysteine required as long as 5 min. In these cases, the number of cysteines that reacted in 5 min is indicated (Table 4).

When primase or zinc-reconstituted primase was denatured in guanidinium chloride, all seven cysteines reacted rapidly with DTNB (Table 4). Since only two cysteines had been DTNB-reactive in the native state, this indicated that about five cysteines were inaccessible to DTNB in the native state. These five cysteines probably correspond to the three zinc-chelating cysteines and two of the cysteines located in the carboxyl-terminal half of primase.

Confirming this correspondence was that about three cysteines became reactive upon zinc removal. That is, freshly reduced apoprimase had four, five, or six cysteines that were DTNB-reactive residues compared to only two in native primase. The freshly reduced apoprimase was particularly difficult to quantitate as indicated by the larger standard deviation (Table 4). Nevertheless, the high DTNB-reactivity of apoprimase indicated that the zinc-ligating cysteine residues were readily available to solvent, and to DTNB when the zinc was absent.

When enzymatically inactive apoprimase was tested for cysteine reactivity, it had slightly more than one reactive cysteine in its native state and three or four in its denatured state (Table 4). There appeared to be about two unreactive cysteines in the oxidized apoprimase, suggesting that, in the absence of zinc, the zinc-ligating cysteines formed disulfide bonds.

DISCUSSION

This study was undertaken to establish the role of the zinc in primase, and it was found that the roles of the zinc and its chelating cysteines are entwined. If the zinc participates in the catalytic reaction, then its removal should have lead to inactive apoprimase. It was shown that reduced apoprimase is fully catalytically active, indicating that the zinc does not play a catalytic role. The first hint as to one of its roles came from the observation that storage of the apoprimase resulted in a loss of activity and that full activity could be recovered by the addition of a mild reducing agent. It was then demonstrated that storage of apoprimase for 10 days at 4 °C was long enough to result in inactive apoprimase. During this time, oxygen must have dissolved in the degassed buffer and reacted with the protein to form the observed disulfide bond and inactivate the enzyme. In contrast, primase with zinc did not lose activity during the same period of time. Therefore, one of the roles of the zinc is to prevent disulfide bond formation and concomitant primase inactivation.

Zinc is not particularly redox-active and is relatively biologically abundant. Zinc probably plays an antioxidant role in addition to its structural role in many zinc proteins and enzymes. It can be used simultaneously as a structural element to bring its ligating residues into close proximity, to orient those residues and their adjacent secondary structures, and to prevent the subsequent oxidation of ligated cysteines.

Zinc plays a role in the function of many enzymes (Coleman, 1992). Zinc has a low energy barrier between coordinating four, five, or six total ligands, and, when used for catalysis, one of the exchangeable ligands is water or hydroxide depending on the pH. In such cases, zinc acts as an electron sink. If a Lewis acid, the zinc is usually held in place by the side chains of cysteine and histidine so that the metal is placed in proximity to a conserved glutamate residue (Vallee & Auld, 1990). Zinc can also play structural roles in protein function such as in the aspartate transcarbamoylase regulatory subunit and T4 SSB (Giedroc et al., 1987; Nadler et al., 1990). In these proteins, the zinc is tetrahedrally coordinated by only cysteines and histidines. The stable ligation by four sulfur and nitrogen atoms somehow prevents

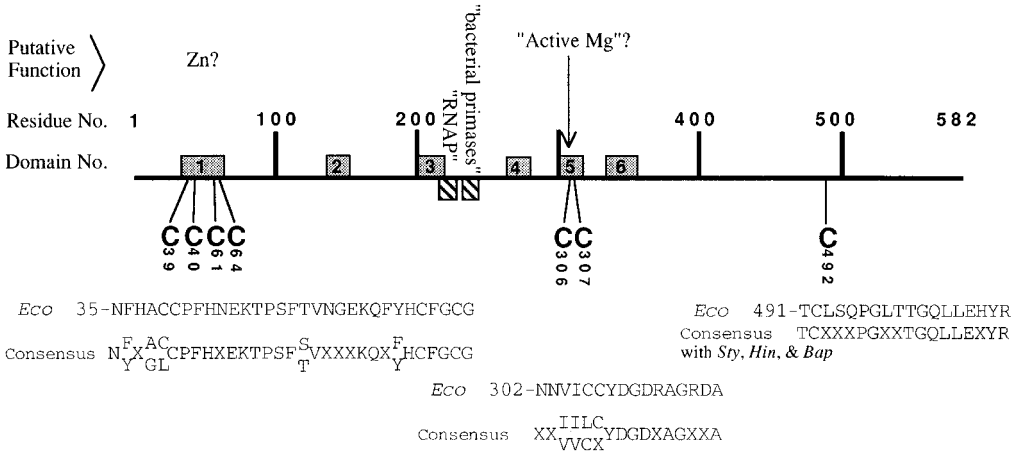

FIGURE 4: Primase sequence and function correlations and the locations of cysteines. There are at least eight conserved regions in bacterial primases, some of which have postulated functions. The six shaded boxes represent conserved sequences identified in a comparison of sequences from three bacterial and four bacteriophage primases (Ilyina et al., 1992) while the two striped boxes represent conserved sequences identified in a comparison of six bacterial and one bacteriophage primase (Versalovic & Lupski, 1993). The full amino acid sequences from eight bacteria were used to obtain the consensus sequences shown: *Bacillus subtilis*, GenBank Accession No. X03897; *Clostridium acetobutylicum*, Z23080; *Escherichia coli*, J01687; *Haemophilus influenzae*, L11044; *Listeria monocytogenes*, U13165; *Myxococcus xanthus*, U20669; *Ricksettsia prowazekii*, M95860; and *Salmonella typhimurium*, M14427. Partial sequences were available for *Buchnera aphidicola*, P32000, and *Lactococcus lactis*, D10168.

its interaction with water. As a consequence, the zinc stabilizes the native structure of the portion of the polypeptide in which the ligating residues are located. Several classes of zinc binding polypeptides exist which serve to generate either protein−protein contacts or sequence-specific RNA or DNA binding (Coleman, 1992) such as in transcription factors and other DNA binding proteins (Miller et al., 1985).

Our data indicate that *E. coli* apoprimase is capable of synthesizing primers in a sequence-specific manner. This result effectively eliminates a role in catalysis for the strongly bound zinc of primase. This result also eliminates a role in maintaining proper protein folding since the apoprimase maintains its active conformation. It is the amino acid side chains and the secondary and tertiary structures they adopt that are important for enzymatic function, not the zinc itself. In support of this is that the C39S T7 primase/helicase mutant (equivalent to *E. coli* cysteine 61) is not capable of site-specific primer initiation (Mendelman et al., 1994) even though the mutant can bind 0.5 equiv of zinc. This strongly indicates that the cysteines are more important for function than the zinc to which they are ligated.

We have demonstrated the interplay between the cysteines of *E. coli* primase and its tightly bound zinc. The cysteines were characterized according to their chemical reactivity using PMPS and DTNB, and they fell into three categories: (1) one or two very reactive cysteines; (2) three cysteines that are ligated to the single zinc, at least two of which become oxidized when not bound to the zinc and, when oxidized, prevent zinc reconstitution and; (3) two or three cysteines of low reactivity perhaps because they are buried within the tertiary structure.

The one cysteine that is most PMPS- and DTNB-reactive is probably cysteine 39. We found that cysteine 39 is most readily labeled by fluorescein-5-maleimide and 6-(iodoacetamido)fluorescein (Griep & Mesman, 1995). This cysteine is located adjacent to other zinc binding residues but is not predicted to be an important residue for ligating the zinc (Figure 4). This does not negate that it may be weakly bound to the zinc. In fact, it is likely that the adjacent zinc may lower the p*K* of cysteine 39 by partially stabilizing its anionic form and thereby making it especially reactive. Even though cysteine 39 is the most chemically reactive, it is not essential for function since other bacterial primases have a leucine in this position, suggesting that hydrophobicity is the conserved feature of this residue. In addition, when this residue is covalently attached to fluorescein-5-maleimide, primase retains full activity.

The second category of reactive cysteines are those bound to zinc. In accordance with the results of Stamford and coworkers (Stamford et al., 1992), we find that primase has one tightly associated zinc. The second through the fourth equivalents of PMPS release all of the zinc from primase, indicating that three cysteines are ligands. Organic mercurials react with the zinc-ligating cysteines of aspartate transcarbamoylase in a highly cooperative fashion such that zinc is released in proportion to the amount of PMPS added (Hunt et al., 1984). This appears to be the case with primase as well since there is stoichiometric release of zinc by PMPS. When the zinc is removed from primase, these three cysteines in apoprimase become fast-reacting with DTNB. Therefore, in native primase, it is the zinc that prevents them from being highly reactive with chemical agents.

Amino acid sequence analysis of bacterial and bacteriophage primases predicts that in *E. coli* primase histidine 43 and cysteines 40, 61, and 64 are ligated to the single zinc (Ilyina et al., 1992). The strongest experimental evidence to support this prediction comes from the analogous T7 primase/helicase (Mendelman et al., 1994). T7 primase/helicase that lacks the putative zinc binding motif does not have any bound zinc. In addition, T7 primase/helicase in which cysteine 36 is mutated to a serine only binds 0.5 equiv of zinc (Mendelman et al., 1994). This cysteine helps coordinate the zinc but is not the only ligand involved in doing so.

The zinc binding portion of bacteriophage T7 primase/helicase is involved in site-specific RNA primer synthesis (Bernstein & Richardson, 1988; Mendelman et al., 1994). This is also likely to be the case for the other primases because, like their sequences, their functions are very similar. For instance, T4, T7, and *E. coli* primases have template

trinucleotide consensus initiation sequences. The T4 primase initiation sequence is either 5′-d(GTT) or 5′-d(GCT) (Cha & Alberts, 1986; Nossal & Hinton, 1987), for T7 it is 5′-d(GTC) (Mendelman & Richardson, 1991), and for *E. coli* it is 5′-d(CTG) (Swart & Griep, 1993; Yoda & Okazaki, 1991). Slightly less specific but similar nevertheless is bacteriophage P4 primase which recognizes the dinucleotide 5′-d(CT) initiating from the thymine (Ziegelin et al., 1993).

The two or three least reactive cysteines must be partially buried in the native structure. Given that most of the other cysteines have been accounted for, these must correlate to residues 306 and 307 within motif 5, the "active magnesium" site, and cysteine 492 in the carboxyl-terminal tail (Figure 4). The weakly bound zinc that we identified must be bound to the "active magnesium" site (Ilyina et al., 1992; Pansegrau & Lanka, 1992) because it was easily displaced by either magnesium or dilution. The two cysteines in motif 5 are likely responsible for the binding of the weakly bound zinc at this site. It has been well-established that divalent cations such as cobalt, nickel, zinc, and manganese can be the active metal in other polymerases (Sirover & Loeb, 1976), so it is not that unexpected to find that zinc can bind to the active magnesium site of primase.

The final cysteine in *E. coli* primase falls within the carboxyl terminus but is not highly conserved and is not necessary for function. Mutated *E. coli* primase which lacks these carboxyl-terminal residues functions as a primase but does not interact with DnaB helicase (Tougu et al., 1994) and interacts poorly with SSB (Sun et al., 1994). Thus, the role of the carboxyl terminus seems to be to interact with these other proteins. The carboxyl termini of only four of the bacterial primases have a cysteine at this location. The other five primases have similar numbers of residues, but if they have cysteine in their terminus, then the cysteine is located randomly within the sequence (Griep, 1995). The most carboxyl-terminal cysteine in the *E. coli* primase does not appear to be essential for its function.

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