Thomas, D. D., & Stryer, L. (1982) *J. Mol. Biol. 154*, 145–157.
Tracy, P. B., & Mann, K. G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2380–2384.
van de Waart, P., Bruls, H., Hemker, H. C., & Lindhout, T. (1983) *Biochemistry 22*, 2427–2432.
van de Waart, P., Hemker, H. C., & Lindhout, T. (1984) *Biochemistry 23*, 2838–2842.
Weber, G., & Teale, F. W. J. (1957) *Trans Faraday Soc. 53*, 646–655.
Wolber, P. K., & Hudson, B. S. (1979) *Biophys. J. 28*, 197–210.

# Structural and Functional Differences between the Two Intrinsic Zinc Ions of *Escherichia coli* RNA Polymerase†

David P. Giedroc and Joseph E. Coleman*
*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510*
*Received January 7, 1986; Revised Manuscript Received April 9, 1986*

ABSTRACT: DNA-dependent RNA polymerase (RPase) from *Escherichia coli* contains 2 mol of intrinsic Zn(II)/mol of core enzyme ($\alpha_2\beta\beta'$). In techniques analogous to those employed with the Zn(II) metalloenzyme aspartate transcarbamoylase [Hunt, J. B., Neece, S. H., Schachman, H. K., & Ginsberg, A. (1984) *J. Biol. Chem. 259*, 14793–14803], we show that titration of core or holoRPase with 10 or 16 equiv, respectively, of the sulfhydryl reagent *p*-(hydroxymercuri)benzenesulfonate (PMPS) results in the facile release of 1 mol of Zn(II) [B-site Zn(II)] in a reaction totally reversible with the addition of excess thiol provided no metal chelator is present. If ethylenediaminetetraacetic acid (EDTA) is present, reversal of the PMPS–enzyme complex results in formation of a $Zn_1$ RPase [A-site Zn(II)]. This enzyme retains full transcriptional activity relative to $Zn_2$ RPase on both calf thymus (nonspecific) and T7 ($\sigma$-dependent, specific) DNA templates. If the core enzyme–PMPS complex is incubated with a large excess of another metal such as Cd(II) followed by thiol treatment, a hybrid $Zn_ACd_B$ RPase is formed. Direct treatment of the enzyme with excess Cd(II) also gives rise to a hybrid $Zn_ACd_B$ RPase. Transcription by these enzymes is also comparable to that of the starting $Zn_2$ enzyme. Isolation of in vivo synthesized $Co_2$ RPase and $Cd_2$ RPase and treatment of either enzyme with PMPS/EDTA results in formation of a $Co_A$ and $Cd_A$ enzyme, respectively. $Co(II)_A$ and $Cd(II)_A$ enzymes show 123 and 76%, respectively, of the elongation rates on T7 DNA observed for the Zn(II) enzyme. Visible absorption spectroscopy of the $Co_2$ enzyme exhibits four d–d transition bands positioned at 760 ($\epsilon$ = 800), 710 ($\epsilon$ = 900), 602 ($\epsilon$ = 1500), and 484 ($\epsilon$ = 4000) nm. In addition, two charge-transfer bands are found at 350 ($\epsilon$ = 9600) and 370 ($\epsilon$ = 9500) nm. Only the Co(II) ion bound at site A is associated with this unique set of intense d–d transitions. The positions and intensities of both the visible and charge-transfer bands of $Co(II)_A$ RPase approximate those shown by Co(II)-substituted metalloenzyme sites where the ligands are four S rather than mixed S,N or S,O sites.

DNA-dependent RNA polymerase (RPase)[1] from *Escherichia coli* is a complex, multisubunit Zn(II) metalloenzyme ($M_r \approx 450000$) consisting of the subunit arrangement of $\alpha_2\beta\beta'\sigma$ [review by von Hippel et al. (1984)]. Subunit $\sigma$ confers upon the catalytically competent core enzyme promoter specific recognition in the transcription of RNA chains (Chamberlin, 1974). More limited information exists concerning the role that each of the subunits plays in the transcription process with the possible exception of the $\beta$ subunit (Yura & Ishihama, 1979; Zillig et al., 1976). Mutant forms of the enzyme insensitive to the initiation inhibitor rifamycin without exception contain base changes in the coding sequence for the $\beta$ subunit (Rabussay & Zillig, 1969; Iwakura et al., 1973). Thus, the $\beta$ subunit almost certainly plays a role in the initiation of RNA chains. The $\beta'$ subunit, on the other hand, appears to contribute to template binding due perhaps to its strongly positive charge at neutral pH (Zillig et al., 1970). Topological experiments by Meares and co-workers suggest that the path of the growing RNA chain occurs proximate to both $\beta$ and $\beta'$ subunits with the involvement of $\sigma$ subunit only through two to three rounds of nucleotide incorporation (Hanna & Meares, 1983a,b). Chemical cross-linking experiments between RPase subunits (Hillel & Wu, 1977) and between the enzyme and promoter-containing DNA (Park et al., 1980) are consistent with this picture. None of the individual subunits alone, nor a $\beta$–$\beta'$ heterodimer, possesses significant transcriptional activity (Yarbrough & Hurwitz, 1974).

The precise role, structural or catalytic, played by the two intrinsic Zn(II) ions in the transcription process is not well-defined. Limited data provided by complete denaturation of RPase, isolation of denatured subunits, and renaturation and assessment of Zn(II) binding by individual subunits suggest that one Zn(II) is associated with the $\beta'$ subunit, while the other is either bound by the $\beta$ subunit or present at the $\beta$–$\beta'$ interface (Wu et al., 1977; Miller et al., 1979; Chatterji &

† This work was supported by Grants AM09070-21 and GM21919-11 from the National Institutes of Health. D.P.G. is the recipient of NIH Postdoctoral Fellowship GM10972.

[1] Abbreviations: PMPS, *p*-(hydroxymercuri)benzenesulfonate; PAR, 4-(2-pyridylazo)resorcinol; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; TNG buffer, 10 mM Tris-HCl, pH 7.9, 0.2 M NaCl, and 5% glycerol; RPase, RNA polymerase; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; M, metal.

0006-2960/86/0425-4969$01.50/0 

Wu, 1982a, Chatterji et al., 1984; Solaiman & Wu, 1984, 1985).

Isolation and characterization of a $Co_2$ RPase synthesized in vivo showed only subtle differences in catalytic and biophysical properties compared to the $Zn_2$ enzyme (Speckhard et al., 1977). Spectroscopic studies on a $Co_1Zn_1$ hybrid implicated the bound Co(II) as directly involved in binding the initiating nucleotide and thus integral to the catalytic mechanism (Chatterji & Wu, 1982b; Chatterji et al., 1984). Mechanisms of enzymatic polymerization of both RNA and DNA are not uniformly Zn(II) dependent, since *E. coli* DNA polymerase (Mildvan & Leob, 1979) and T7 RNA polymerase (Coleman, 1974), previously believed to contain intrinsic Zn(II), have recently been shown not to contain Zn(II) in cloned and overproduced preparations (Walton et al., 1982; King et al., 1986).

To avoid potential ambiguities inherent in complete denaturation and reconstitution of a large multimeric enzyme such as RPase, we sought more gentle methods to exchange the intrinsic metals with those possessing more useful spectroscopic properties, such as Cd(II) and Co(II). In this paper, we present a nondenaturing methodology that results in the formation of $Zn_1$, $Co_1$, and $Cd_1$ enzymes in addition to a $Zn_1Cd_1$ metal hybrid enzyme. The separate spectroscopic properties of the two metal binding sites for Co(II) are distinguished. The potential respective functions of both Zn(II) ions in RPase function are discussed.

## Materials and Methods

*Materials.* Ribonucleoside triphosphates, PMPS,[1] and heparin (grade I) were obtained from Sigma Chemical Co. while PAR was from Eastman Kodak Corp. Chelex 100 (200–400 mesh), Bio-Gel A-5m, and Bio-Rex 70 resins were purchased from Bio-Rad Laboratories. DEAE-Sephadex (A-25) was from Pharmacia while phosphocellulose P-11 was obtained from Whatman. [$^3$H]ATP, [$\alpha$-$^{32}$P]UTP, and $^{65}ZnCl_2$ were from New England Nuclear Corp. while $^{115}CdCl_2$ was purchased from Amersham Corp. DNA from calf thymus was a Calbiochem product while T7 DNA was obtained as described (King et al., 1986). Heparin–agarose was prepared as outlined by Davison et al. (1979). Stock solutions of Tris-HCl and NaCl were routinely subjected to passage through Chelex X-100 to reduce the level of Zn(II) contamination.

*Preparation of RPase.* RPase was purified from *E. coli* 1200 cells (grown in yeast/tryptone broth to $\sim^3/_4$ log stage) by the method of Burgess and Jendrisak (1975) through the Bio-Gel A-5m step. Further chromatography on phosphocellulose in the presence of 50% glycerol yielded purified holoRPase and core enzyme (Gonzalez et al., 1977). With the quantitative assay of Chamberlin et al. (1979) (described below), the fraction of active molecules varied from 25 to 50% in multiple preparations, typical of values in the literature from this assay. A $\sigma$ subunit preparation was obtained by chromatography of RPase isolated from the Bio-Gel column, on Bio-Rex 70 in series with a DEAE column (Lowe et al., 1979). The $\sigma$ fraction was approzimately 60% pure as judged by SDS–PAGE electrophoresis.

*Isolation of in Vivo Synthesized $Co_2$ and $Cd_2$ RPases. E. coli* 1200 cells were grown in M63 minimal media supplemented with $5 \times 10^{-6}$ M $CoCl_2$ or $CdCl_2$ at 37 °C as described by Speckhard et al. (1977). To reduce trace contamination of Zn(II) to levels less than $5 \times 10^{-8}$ M, the potassium phosphate component of the media was treated with Chelex 100, while all growth flasks were acid-washed and exhaustively rinsed with metal-free water. In all cases, cells were first grown in 100 mL of minimal media supplemented with the indicated metal ion. After several serial 100-mL cultures, growth was then scaled up to 1-L glass carboys or to a 10-L fermentor. Cells were isolated when stationary phase was reached. For small-scale isolation of RPase (10–15 g of cells), a method employing heparin–agarose was utilized on the basis of those described by Chamberlin et al. (1983) and Davison et al. (1979). The RPase was further purified by gel filtration chromatography and/or phosphocellulose in 50% glycerol.

*RNA Polymerase Assay.* For routine assays, 100-$\mu$L reaction mixes contained 40 mM Tris-HCl, pH 7.9, 20 mM $MgCl_2$, 10 mM $\beta$-mercaptoethanol, 0.35 mM EDTA, and 0.4 mM each of [$^3$H]ATP, CTP, GTP, and UTP. [$\alpha$-$^{32}$P]UTP was often used in place of [$^3$H]ATP, while 20 $\mu$g/mL bovine serum albumin was present in some assays. Typically, 1–2 $\mu$g of RPase (22–44 nM) and 50 $\mu$g/mL calf thymus DNA or 78 $\mu$g/mL T7 DNA (3 nM) were present unless otherwise noted. Exogenous $\sigma$ subunit was added to core enzyme preparations as indicated in the figure legends and tables. Reactions were initiated by transfer to a 37 °C water bath, allowed to proceed for 10 min, and terminated by placement on ice. To determine the amount of acid-insoluble RNA, an aliquot of each reaction mix (45–65 $\mu$L) was spotted onto 3MM (Whatman) filter discs (prewetted with 5% trichloroacetic acid) washed, dried, and subjected to liquid scintillation counting (King et al., 1986).

For determination of specific rates of elongation by RPase on T7 DNA with the quantitative assay of Chamberlin et al. (1979), 1-mL reaction mixes were prepared as described (Chamberlin et al., 1979), and 100-$\mu$L aliquots were removed at 2, 3, 4, 5, and 6 min following initiation of transcription at 30 °C and analyzed for both acid-precipitable RNA and transcript length. RNA transcripts produced per unit time were subjected to electrophoresis on 37 × 16 × 0.02 cm 0.5% agarose/1.75% polyacrylamide [0.08% in bis(acrylamide)] gels in 40 mM Tris-HCl, 20 mM sodium acetate, and 2 mM EDTA, pH 7.5, as described by Chamberlin et al. (1979). Average transcript sizes were determined by comparison with the migration of major RNA transcripts produced by T7 RPase on T7 DNA (Golomb & Chamberlin, 1974). The basis of this assay is that under the ionic conditions employed all molecules that are able to do so will initiate chains at the A1–A3 promoters and begin elongation in the first 90 s at 30 °C. Heparin (100 $\mu$g/mL) is then added to prevent reinitiation. Elongation continues at a constant rate for native RNA polymerase (17 $s^{-1}$) until a strong terminator is encountered at 7720 bp, which takes about 6–8 min. The specific activity is determined as the amount of [$\alpha^{32}$P]UMP incorporated into RNA per minute per milligram of RPase added. A maximum value of 550 nmol $min^{-1}$ $mg^{-1}$ was estimated from the observed elongation rate, 17 $s^{-1}$, determined by Chamberlin et al. (1979), if 100% of the molecules could initiate at one of the three promoters. A specific activity less than 550 provides an approximation of the percent molecules in a preparation capable of initiating and elongating RNA chains. Typical RPase preparations following literature methods contain 25–50% active molecules as accessed by this assay.

*Reaction of RNA Polymerase with PMPS.* RPase was dialyzed into metal-free 10 mM Tris-HCl, pH 7.9, 0.2 M NaCl, and 5% glycerol (TNG buffer) to remove thiol and EDTA. PMPS was added from a stock solution (4 mM based on weight) prepared in distilled $H_2O$ or 50 mM Tris-HCl, pH 7.9, and an aliquot of enzyme was assayed by the standard assay except that $\beta$-mercaptoethanol was omitted. Zn(II) released by reaction of the enzyme with PMPS was determined

at 23 °C in quartz cuvettes (0.75–1.0 mL) with $1 \times 10^{-4}$ M 4-(2-pyridylazo)resorcinol (PAR) and 1.5–2.5 μM core RPase or holoRPase in TNG buffer. The formation of the Zn(II)–$PAR_2$ complex was monitored at 500 nm and converted to moles of Zn(II) released with $\epsilon_{500} = 6.6 \times 10^{-3}$ $M^{-1}$ $cm^{-1}$ for Zn(II)–$PAR_2$ (Hunt et al., 1984). For large-scale treatment of RPase with PMPS, 12 or 35 equiv of the reagent was added to enzyme samples dialyzed into TNG.

PMPS was in contact with the enzyme for 10 min at 23 °C after which time the reaction was reversed with 1 mM DTT followed by dialysis. For those samples containing excess Cd, sequential dialyses were required against TNG, 10 mM EDTA, and 1 mM DTT followed by TNG, 1 mM EDTA, 10 mM DTT, and 1 mM *o*-phenanthroline and finally TNG, 0.1 mM EDTA, and 0.1 mM DTT, each for 24 h at 4 °C. As a control, an untreated RPase sample (or that treated with PMPS in the absence of EDTA) was carried through the same manipulations.

*Circular Dichroism Spectroscopy.* CD spectra were determined at 23 °C on core RPase solutions (0.1–0.2 mg/mL in 10 mM Tris-HCl, pH 7.9, 0.2 M NaCl, 0.08 mM EDTA, 0.08 mM DTT, 5% glycerol) over a 0.2-cm path on a Cary 61 circular dichrograph operating at 50 millideg full scale. Deviation between repeat scans at 220 nm was typically 1 millideg.

*Absorption Spectroscopy.* Visible absorption spectra were collected on enzyme solutions (0.3–0.4 mL, 3–8 mg/mL) in masked quartz cuvettes with a Cary 219 spectrophotometer. Spectra obtained for Co(II) enzymes were converted to spectra of the Co(II) chromophores alone by subtracting the contributions from the ultraviolet absorption and scattering by the same concentration of the native Zn(II) enzyme.

*Protein Concentration.* RPase concentrations were determined with $A_{280}^{0.1\%} = 0.65$ for holoRPase and 0.58 for core enzyme (Burgess, 1976). $A_{280}$ values were corrected for light scattering. Concentrations were also determined by the Coomassie blue binding assay (Bradford, 1976) with bovine serum albumin as the standard; satisfactory agreement (±10% of expected values based on $A_{280}$) was typically obtained.

*Atomic Absorption.* Zn(II) content was determined by flame atomic absorption on an Instrumentation Laboratories Model 157 atomic absorption spectrometer. Cd(II) and Co(II) values were obtained with a graphite furnace attachment with the pyrolysis and atomization temperature protocols set as recommended by the manufacturer. Enzyme samples analyzed for Cd(II) were diluted into 0.1% $HNO_3$, and 10 μL was subjected to analysis, while Co(II) samples were first dialyzed into distilled $H_2O$ and 40 μL was analyzed.

## Results

*Characterization of Reaction of PMPS with RPase.* Sulfhydryl reagents are known to inhibit RPase (Harding & Beychok, 1973; Yarbrough & Wu, 1974). Figure 1 compares the concentration dependence of the inhibition of core enzyme by PMPS when transcribing on calf thymus DNA (panel A), of core enzyme when supplemented with σ subunit transcribing on T7 DNA (panel B), and of holoenzyme transcribing on T7 DNA (panel C). The number of equivalents of PMPS required for 50% inhibition is 6–8, while 12–14 mol of PMPS/mol of RPase are required to totally inhibit. These results are similar to those previously noted with the related compound PCMB (Harding & Beychok, 1973). Reversal of the PMPS–enzyme reaction with β-mercaptoethanol results in nearly complete restoration of the original enzymic activity. Recovery of activity is similar in the presence or absence of the chelator EDTA.

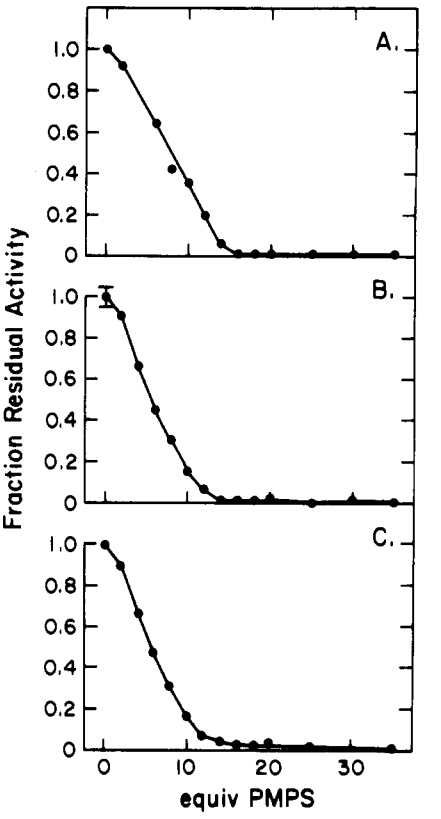


FIGURE 1: Inhibition of RPase activity during a titration with PMPS. (A) Core RPase (0.65 μM) in TNG buffer was titrated over time with successive aliquots of a concentrated stock solution of PMPS to give the indicated number of equivalents relative to RPase. At 10 min after each addition, an aliquot of enzyme was assayed for activity on calf thymus DNA as described under Materials and Methods. The residual activity is expressed as a fraction of the activity observed before the addition of PMPS. (B) The same experiment as in (A) was carried out except that core RPase supplemented with exogenous σ to 70% maximal stimulation (to give 0.53 μM holoRPase) was titrated with PMPS and the activity assessed on T7 DNA. (C) The same as in (B) except that purified holoRPase (0.44 μM) was used with the activity determined on T7 DNA. In each case, after the final addition of PMPS, separate aliquots were treated with 3.5 mM β-mercaptoethanol with and without 2 mM EDTA. These measurements represent recovered activity, which varied between 60 and 90% following the 2.5-h incubations above; in all cases, similar recoveries were noted in the presence and absence of EDTA.

Studies on the titration of another Zn(II) metalloenzyme, aspartate transcarbamoylase (ACTase; subunit composition $c_6r_6$), with PMPS showed that the organomercurial promotes sequential release of each of the six Zn(II) ions bound to four sulfhydryl groups in each of the six r chains. This reaction induces dissociation of the catalytic (c) and regulatory (r) subunits. The release of Zn(II) from ATCase can be monitored by complex formation between extruded Zn(II) and the high-affinity indicator dye 4-(2-pyridylazo)resorcinol (PAR). Zn(II)–$PAR_2$ exhibits an absorption maximum at 500 nm (Hunt et al., 1984). Figure 2A illustrates the formation of a complex between PAR and Zn(II) as core RPase is titrated with PMPS. The reaction is linear up to a level of 10 equiv of PMPS, while further addition of PMPS, 35–50 mol equiv, gives rise to no additional change in the amount of Zn(II)–$PAR_2$ complex formed. Four different core enzyme preparations showed 0.98 ± 0.13 equiv of Zn(II) to react with PAR. The remaining mole of Zn(II) in the enzyme does not react even after a 17-h incubation with excess PMPS.

Figure 2B demonstrates that when purified holoRPase is used in place of core enzyme, a somewhat greater number of mole equivalents of PMPS is required before the maximum

Table I: Metal Content and Enzymatic Activity on Calf Thymus DNA of Native and PMPS-Treated Core RPases

| enzyme + treatment[a] | Zn(II) (mol) | Cd(II) (mol) | sp act. [nmol of AMP incorp (10 min)$^{-1}$ (mg of protein)$^{-1}$] | recovered activity |
|---|---|---|---|---|
| core RPase | 1.7 ± 0.15 | | 748 ± 56 | 100 |
| core RPase[b] | 1.77 | | 841 | 112 |
| core RPase + PMPS + thiol | 1.8 ± 0.1 | | 793 | 106 |
| core RPase + PMPS + EDTA + thiol | 1.0 ± 0.1 | | 729 | 97 |
| core RPase + PMPS + Cd(II) | 0.86 | 0.92 ± 0.11 | 761 | 102 |
| core RPase + Cd(II) | 1.01 | 0.84 | 782 | 105 |

[a] Enzyme preparations are treated with PMPS etc. as described under Materials and Methods. [b] Untreated RPase carried through the exhaustive dialysis procedure outlined for PMPS-treated samples as described under Materials and Methods.

Table II: Metal Content and Enzymatic Activity on T7 DNA of Native and PMPS-Treated Core and HoloRPases

| enzyme + treatment[a] | Zn(II) (mol) | Cd(II) (mol) | designation | sp act. [nmol of UMP incorp min$^{-1}$ (mg of protein)$^{-1}$] (% act. recovered)[b] |
|---|---|---|---|---|
| core RPase | 1.85 | | $Zn_2$ | 137 (100%) |
| core RPase + PMPS + EDTA + thiol | 1.08 | | $Zn_A$ | 115 (84%) |
| core RPase + PMPS + Cd(II) | 0.86 | 0.92 ± 0.11 | $Zn_ACd_B$ | 145 (106%) |
| holoRPase | 2.16 | | $Zn_2$ | 190 (100%) |
| holoRPase + PMPS + EDTA + thiol | 0.99 | | $Zn_A$ | 191 (100%) |

[a] Enzyme preparations treated as outlined under Materials and Methods. [b] Determined with the quantitative assay of Chamberlin et al. (1979). Core enzyme was supplemented with exogenous $\sigma$ subunit to give rise to ≈85% maximal stimulated activity. The absolute activities of native core RPase + $\sigma$ and holoRPase above cannot be strictly compared since one preparation was not derived from the other.

absorbance is reached; however, 1.0 mol of Zn(II) is also released from this enzyme (Table I). Thus, the presence of $\sigma$ subunit has little effect on the release of Zn(II) from RPase by PMPS.

We have designated the "PMPS-removable" Zn(II) as B-site Zn(II) and the "PMPS-insensitive" Zn(II) as A-site Zn(II). Reversal of the core RPase–PMPS complex with thiol must dissociate the Zn(II)–$PAR_2$ complex, since a $Zn_2$ RPase is reconstituted containing 1.8 mol of Zn(II)/mol (Table I). However, if the RPase–PMPS complex is treated with EDTA prior to thiol reversal, only 1.0 mol of Zn(II) is found in this enzyme (Table I). These series of steps thus provided us with a means of preparing $Zn_A$ RPase with quantitative recovery of transcriptional activity relative to the starting $Zn_2$ enzyme (Table I).

Given the observation that PMPS significantly perturbs the environment about one of the two intrinsic Zn(II) ions, it was reasoned that the exchange of this removable Zn(II) site with other metals would be possible. Indeed, it was found that a large excess of Cd(II) incubated with the RPase–PMPS complex followed by reversal of the complex with thiol and exhaustive dialysis gives rise to 1 mol of Cd(II) stably integrated into the enzyme presumably at the B site. A large excess of Cd(II) presented to RPase in the absence of thiol also results in a stable $Zn_ACd_B$ hybrid RPase, suggesting that formation of the enzyme–PMPS complex is not an obligatory intermediate in the Cd(II) exchange reaction (see below).

*Catalytic Properties of $Zn_A$, $Zn_ACd_B$, and $Zn_2$ RPases.* The metal contents of enzymes prepared as described above and their transcriptional activities on calf thymus DNA are given in Table I. All enzyme species give approximately identical activities relative to the native ($Zn_2$) enzyme. The activities of the same core enzymes supplemented with $\sigma$ and transcribing from a $\sigma$-dependent promoter are shown in Table II and compared to the activities of $Zn_A$ and $Zn_AZn_B$ holoenzyme preparations. The slightly lower activities of the enzymes derived from core reflect undersaturation with $\sigma$ (see complete titration in Figure 3 below). Finally, no significant difference in the ability of any of these metallo RPases to locate a promoter and utilize the strong termination sequence at 7720 bp on T7 DNA was noted (data not shown).

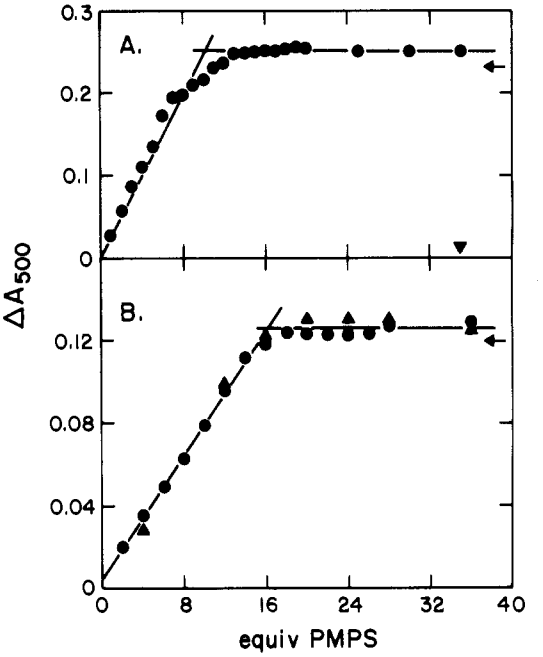


FIGURE 2: Zn(II) release from RPase during a titration with PMPS. (A) Core enzyme (3.5 $\mu$M) in 1.0 mL of TNG buffer containing 0.1 mM PAR was titrated at ambient temperature over time with successive additions of stock PMPS to give the indicated number of equivalents relative to RPase. Following the addition of the reagent, the absorbance at 500 nm was monitored until constant and then recorded. The spectrophotometer was adjusted to zero absorbance units prior to the first addition of PMPS such that subsequent readings represent $\Delta A_{500nm}$. The arrow represents the expected change in absorbance if 1.0 mol of Zn(II) is released from the enzyme. (▼) Absorbance of the same sample after reaction with 1 mM DTT. (B) A similar experiment was carried out as in (A) except that 1.82 $\mu$M holoRPase in 0.7 mL of TNG and 0.1 mM PAR was titrated with succesive aliquots of stock PMPS in replicate experiments. The maximum average absorbance represents 1.05 mol of Zn(II) complexed by PAR.

Titration of $Zn_A$ and $Zn_2$ core enzymes with $\sigma$ subunit reveals nearly identical abilities of the activator to enhance promoter-specific transcription of both enzymes on T7 DNA (Figure 3A). Titration of a fixed amount of T7 DNA with

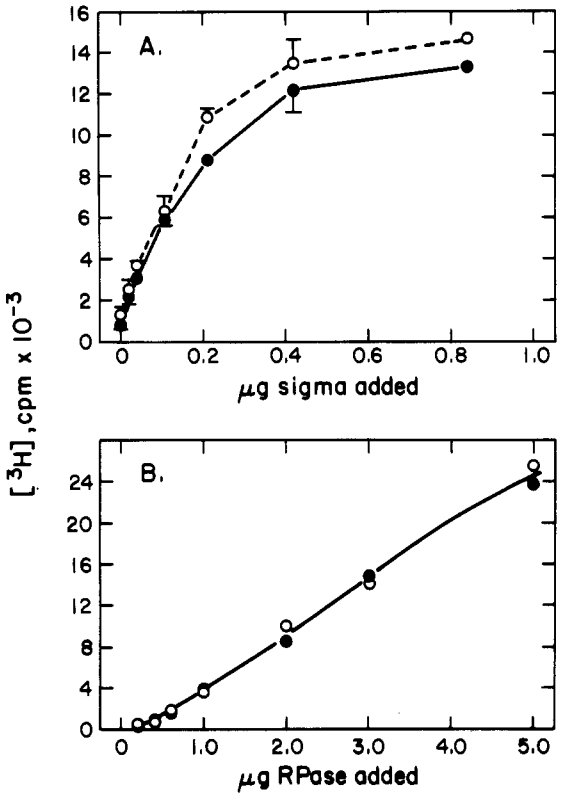


FIGURE 3: (A) Stimulation of transcription by $Zn_A$ and $Zn_2$ core RPases on T7 DNA by exogenous $\sigma$ subunit. A total of 1.0 $\mu$g of $Zn_A$ RPase (○) or $Zn_2$ RPase (●) was incubated with the indicated amount of partially purified $\sigma$ preparation and subjected to assay in duplicate on T7 DNA. The ordinate indicates the cpm incorporated into RNA as described under Materials and Methods. (B) Titration of T7 DNA with $Zn_A$ and $Zn_2$ core RPases supplemented with a constant ratio of $\sigma$. A total of 7.8 $\mu$g of T7 DNA was incubated with the indicated amounts of $Zn_A$ RPase (○) and $Zn_2$ RPase (●) supplemented with 0.32 $\mu$g of $\sigma/\mu$g of enzyme (≈85% maximal stimulation; cf. panel A). The ordinate is the same as panel A. At the indicated concentrations of enzyme and DNA, the range of RPase:T7 DNA molar ratios varied from 2.3 to 50.

$Zn_A$ and $Zn_2$ enzymes supplemented with a constant ratio of $\sigma$ shows identical rises in AMP incorporation, suggesting that the ability to utilize specific promoter sequences is not significantly affected by the loss of $Zn_B$. In other words, neither the rate of transcription nor the binding of enzyme to a specific promoter appears dependent on $Zn_B$.

Since RPase preparations typically exhibit a significant fraction of molecules unable to initiate RNA chains as judged by the quantitative assay (Chamberlin et al., 1979), it was necessary to investigate whether the specific activity of the starting preparation affected the ability of PMPS to give rise to an enzyme population with 1 mol of Zn(II); i.e., do inactive and active molecules exhibit differential reactivities to PMPS? For example, a preparation with 50% active molecules all of which contain ~2 mol of Zn(II) could, after reaction with PMPS/EDTA, result is a heterogeneous population containing 50% inactive apoRPase as a result of PMPS treatment and 50% $Zn_2$ active molecules unaffected by PMPS. If this were the case, the starting activity would significantly effect the resulting metal stoichiometries obtained following treatment with PMPS/EDTA. As shown in Table III, enzyme preparations with specific activities ranging from 3 to 50% active molecules exhibit ~2 mol of Zn(II) and give rise to preparations with ~1 mol of Zn(II) following PMPS/EDTA treatment. Thus, inactive and active molecules alike appear to undergo Zn(II) release by PMPS.

Table III: Metal Content and Enzymatic Activity on T7 DNA of Various HoloRPase Preparations prior to and following PMPS/EDTA Treatment

| holoRPase preparation | sp act. [nmol of UMP incorp $min^{-1}$ (mg of protein)$^{-1}$][a] | Zn(II) (mol) | Zn(II) following PMPS/EDTA treatment |
|---|---|---|---|
| 1 | 16 | 1.98 | 1.13 |
| 2 | 35 | 2.10 | 1.00 |
| 3 | 126 | 1.8 ± 0.1 | 1.0 ± 0.1 |
| 4 | 192 | 2.10 | 1.00[b] |
| 5[c] | 255 | 1.85 | 1.08 |

[a] Determined with the quantitative assay of Chamberlin et al. (1979). A specific activity of 550 indicates 100% functional RPase molecules in a preparation (Chamberlin et al., 1979); the above preparations vary from ≈3 to 46% active molecules. [b] Reflects Zn(II) content of exhaustively dialyzed RPase–PMPS complex prior to thiol reversal. [c] This preparation was assayed and chromatographed on Bio-Rex 70, and the core enzyme was collected and treated with PMPS/EDTA.

Table IV: Molar Ellipticities at 221 nm of Various Metal-Substituted Core RPases

| core RPase[a] | $[\theta]$ (deg $cm^2$ $dmol^{-1}$) |
|---|---|
| $Zn_2$ | −10300 |
| $Zn_A$ | −10051 |
| $Zn_ACd_B$ | −9325 |
| $Cd_2$ | −10529 |

[a] Metal stoichiometries for $Zn_2$, $Zn_A$, and $Zn_ACd_B$ enzymes are indicated in Table II while that for the $Cd_2$ enzyme is given in Table V.

*Circular Dichroism Spectroscopy of $Zn_A$, $Zn_ACd_B$, and $Zn_2$ Core RPases.* To be sure that no major conformational changes occurred during the various treatments of the enzyme, near-UV circular dichroic spectra were obtained for $Zn_A$, $Zn_ACd_B$, and $Zn_2$ core enzymes. All exhibited two negative extrema at 206 and 221 nm with the ratio of the values of their molar ellipticities essentially unchanged (spectra not shown). All enzymes exhibited similar molar ellipticities at 221 nm (Table IV). The $[\theta]$ value observed for $Zn_2$ (native) core RPase is in good agreement with the value of 10500 deg $cm^2$ $dmol^{-1}$ reported by Levine et al. (1980). The $[\theta]$ value for the biosynthesized $Cd_2$ core enzyme (see below for preparation) is also given in Table IV and is identical with that of the $Zn_2$ enzyme.

*Investigation of Metal Exchange Properties of $Zn_2$, $Cd_2Zn_2$–PMPS, and $Zn_A$ RPases.* Extended dialysis of native *E. coli* RPase against 1–10 mM EDTA for 72 h fails to remove any intrinsic Zn(II) from the enzyme (cf. Table I) (Wu et al., 1977). To examine in more detail the metal exchange behavior of both native and PMPS-complexed enzymes, we employed the equilibrium binding protocol of Coleman and Vallee (1960). The presence of $^{65}Zn$(II) at 100- and 2000-fold mol/mol excess results in only partial exchange at one site, presumably B site (Table V). When the RPase–PMPS complex is treated with a 200-fold mole excess of $^{65}Zn$(II), atomic absorption analysis indicates 100% recovery of Zn(II) in the final product (i.e., ~2 mol of Zn(II)). However, only 0.16 mol of $^{65}Zn$(II) is present in the final enzyme (Table V). The low but significant level of $^{65}Zn$(II) incorporation presumably represents very slow exchange at the PMPS-modified site. At a 2000-fold mole excess of $^{65}Zn$(II), a significantly greater amount of the exogenous metal, i.e., approaching ~1 mol/mol, is incorporated into the final $Zn_2$ enzyme (Table V). As expected, if the RPase–PMPS complex is dialyzed against 1 mM EDTA and exogenous $^{65}Zn$(II), the final product shows ~1 mol of Zn(II) and no $^{65}Zn$(II) incorporation. Both $Zn_2$

Table V: Metal Exchange Properties of Various Metal-Substituted RPases[a]

(A) $^{65}Zn(II)$ Exchange

| enzyme | x-fold excess | Zn(II) before dialysis (mol) | after dialysis (mol) | | % activity retained after $^{65}Zn$ dialysis |
|---|---|---|---|---|---|
| | | | $^{65}Zn(II)$ | Zn(II) | |
| $Zn_2$ core | 100 | 1.71 | 0.27 | 1.67 | |
| $Zn_2$ holo | 2000 | 1.87 | 0.59 | 1.77 | |
| $Zn_2$ holo–PMPS[b] | 200 | 1.73 | 0.16 | 1.74 | 65[c] |
| $Zn_2$ holo–PMPS | 2000 | 1.87 | 0.83 | 1.89 | |
| $Zn_2$ holo–PMPS + EDTA[d] | 200 | 1.73 | 0 | 0.83 | 66[e] |

(B) $^{115}Cd(II)$ Exchange

| enzyme | x-fold excess | before dialysis (mol) | | after dialysis (mol) | | |
|---|---|---|---|---|---|---|
| | | Zn(II) | Cd(II) | Zn(II) | $^{115}Cd(II)$ | Cd(II) |
| $Zn_2$ holo | 2000 | 1.87 | nd[f] | 0.88 | 0.60 | 0.85 |
| $Zn_2$ holo–PMPS | 200 | 1.73 | nd | 0.89 | 0.34 | 0.49 |
| $Cd_2$ core | 250 | 0.3 ± 0.1 | 1.7 ± 0.2 | 0.46 | 0.89 | 1.49 |
| $Cd_2$ core | 2600 | 0.3 ± 0.1 | 1.7 ± 0.2 | 0.20 | 0.77 | 1.55 |

(C) $^{65}Zn(II)$ Binding

| enzyme | x-fold excess | Zn(II) before dialysis (mol) | after dialysis (mol) | | % activity retained after $^{65}Zn$ dialysis |
|---|---|---|---|---|---|
| | | | $^{65}Zn(II)$ | Zn(II) | |
| $Zn_A$ core | 100 | 1.00 | 0.20 | 1.15 | |
| $Zn_A$ holo | 200 | 0.90 | 0.17 | nd | 75 |
| $Zn_A$ holo + DTT[g] | 200 | 0.90 | 0.18 | nd | |

[a] Carried out as described (Coleman & Vallee, 1960) on 0.9–1.0-mL aliquots of enzyme (1.0–2.5 μM in TNG) dialyzed against 100 mL of TNG supplemented with the appropriate concentration of $^{65}Zn(II)$ or $^{115}Cd(II)$ to give the indicated mole excess of exogenous metal. Once equilibrium was reached, the enzyme samples were placed in fresh TNG and finally dialyzed exhaustively against 2 to 3 changes of TNG and 10 mM EDTA until significant change in protein-associated cpm ceased to occur. Protein and metal analyses were then performed on the dialysis bag contents. [b] A total of 1 mM DTT was added to reverse the RPase–PMPS complex; 0.1 mM DTT was included in all subsequent dialyses. [c] Specific activity (quantitative assay) = 80 ± 14 nmol of UMP incorp $min^{-1}$ $mg^{-1}$ after dialysis. [d] A total of 1 mM EDTA was present in all buffers. [e] Specific activity (quantitative assay) = 81 ± 2 nmol of UMP incorp $min^{-1}$ $mg^{-1}$ after dialysis. [f] nd, not determined. [g] A total of 1 mM DTT present in all buffers.

Table VI: Metal Content and Transcriptional Properties of Various $M_2$ and $M_A$ RPases[a]

| RPase | Zn(II) (mol) | Co(II) (mol) | Cd(II) (mol) | sp act. | | incorp rate (nucleotide $s^{-1}$)[d] |
|---|---|---|---|---|---|---|
| | | | | calf DNA[b] | T7 DNA[c] | |
| $Zn_2$ | 1.70 | | | 622 | 223 | 17.9 ± 0.5 |
| $Zn_A$ | 0.90 | | | 609 | 166 | 16.8 ± 1.8 |
| $Co_2$ | ≤0.1 | 2.0 ± 0.4 | | 318 | 73 ± 13 | 20.8 ± 0.1 |
| $Co_A$ | | 1.0 ± 0.1 | | 265 | 50 | 21.7 |
| $Cd_2$[e] | 0.3 ± 0.1 | | 1.7 ± 0.2 | 168 | 60 ± 5 | 12.6 |
| $Cd_A$[e] | 0.45 ± 0.05 | | 0.55 ± 0.05 | 183 | 64 | 13.3 |

[a] $M_A$ enzymes were prepared from $M_2$ by PMPS/EDTA treatment followed by exhaustive dialysis as described under Materials and Methods. All of the assays using T7 DNA as template presented in this table and elsewhere in this paper represent sequential assays of acid-precipitable $^{32}P$-labeled mRNA resulting from initiation at the $A_1$, $A_2$, and $A_3$ promoters, coupled with identification of the transcript lengths by gel electrophoresis of the labeled transcripts. At times of 2, 4, 5, 6, 8, and 15 min after initiation, aliquots were removed from the reaction mix; 10% was used to determine TCA-precipitable counts and the remainder prepared for gel electrophoresis. The radioautography of the final gels of the transcripts produced at the sequential time points uniformly showed progressive increase in average mRNA length over the first 6 min and then leveled off at the expected average length of 7133 bases expected from initiation at the three A promoters and termination at the terminator for *E. coli* RNA polymerase at 7720 bp on the T7 genome. At least 90% of the mRNA consisted of this transcript after 6–8 min of reaction, with only a small percentage of read-through typical of this terminator. The native $Zn_i$ and the $Zn_A$, $Co_2$, $Co_A$, $Cd_2$, and $Cd_A$ enzymes all showed the same pattern of mRNA development with correct termination. The only differences were the small variations in time development of the mRNA as reflected in the calculated elongation rates. [b] Nanomoles of UMP of incorp $(10\ min)^{-1}$ $(mg\ of\ protein)^{-1}$. Values are representative to 10–15%. [c] Nanomoles of UMP incorp $min^{-1}$ $(mg\ of\ protein)^{-1}$ as determined with the quantitative assay (Chamberlin et al., 1979). Conditions: 2 μg of RPase (44 nM) and 78 μg T7 DNA (3 nM) in 1 mL. [d] Calculated from T7 RNA transcript gels. The uncertainty when given represents the range of two independent experiments. [e] Since these enzymes have significant Zn(II) present in the A site, the observed incorporation rates represent an upper limit.

and $Zn_A$ enzymes exhibit indistinguishable specific activities following extended dialysis against $^{65}Zn(II)$, which represents 65% of the input activity of the native enzyme (Table VA).

Consistent with the data in Table I, equilibrium dialysis experiments with excess $^{115}Cd(II)$ gives rise to an enzyme with 1 mol each of Zn(II) and $^{115}Cd(II)$ (Table V). Likewise, presentation of the PMPS-complexed RPase with nonradioactive Cd(II) prior to thiol reversal results in formation of a $Zn_ACd_B$ hydrid RPase (Table I). When a 200-fold mole excess of $^{115}Cd(II)$ is presented to the $Zn_2$–PMPS complex in a dialysis experiment, the final product also contains ~1 mol of Zn(II) with ~40% of the B sites occupied by $^{115}Cd(II)$. Thus, Cd(II) and the organic mercurial can act independently of one another in displacing the B-site Zn(II) as demonstrated by the ability of exogenous $^{115}Cd(II)$ to exchange with one of the Cd(II) ions in the $Cd_2$ core RPase (Table V).

Given the relatively poor ability of RPase to exchange the B-site Zn(II) with $^{65}Zn(II)$ with or without PMPS, the ability of $Zn_A$ RPase to reassociate with exogenous $^{65}Zn(II)$ was investigated. As shown in Table V, the $Zn_A$ enzyme exhibits little capacity to rebind a second equivalent Zn(II) once a metal-free B site has been present. Examination of the relative activities of the $Zn_A$ enzyme samples following dialysis against $^{65}Zn(II)$ indicates some loss of activity (28%) but not greater than that shown by the $Zn_2$ enzyme under comparable conditions (35% loss) (Table VC).

*Preparation and Properties of $Co_2$ and $Cd_2$ RPases and Their Treatment with PMPS.* *E. coli* 1200 cells were grown on Zn(II)-depleted M63 minimal media supplemented with either $5 \times 10^{-6}$ M $CoCl_2$ or $CdCl_2$ as outlined under Materials

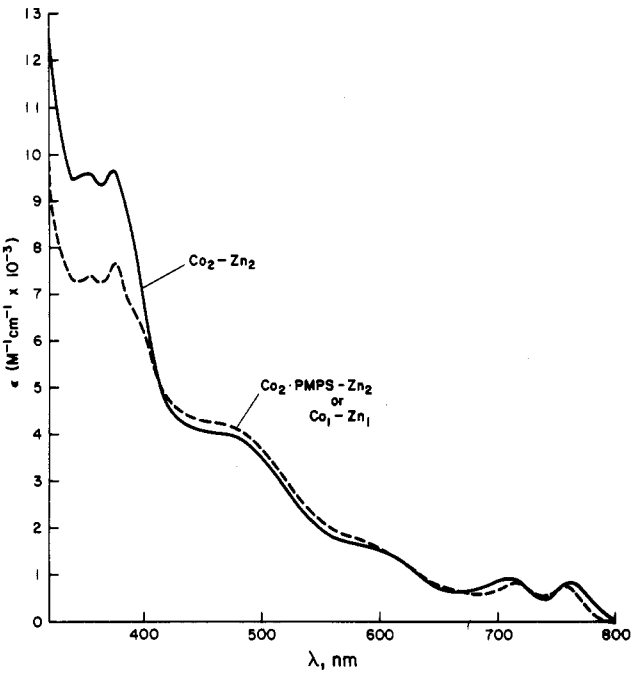


FIGURE 4: Visible absorption spectra of $Co_2$ RPase (8.27 mg/mL) (—) and $Co_2$ RPase treated with 35 equiv of PMPS (– –) minus the spectra exhibited by the same concentration of the $Zn_2$ enzyme in TNG buffer. The molar absorptivities were calculated from the observed absorption and the molar concentration of RPase (assuming a $M_r$ 450000). $Co_2$ RPase was found to contain 1.8 ● 0.1 mol of Co(II). Addition of EDTA followed by thiol did not significantly alter the observed $Co_2$–PMPS – $Zn_2$ spectrum; further, the $Co_A$ – $Zn_A$ difference spectrum is essentially superimposable upon this latter spectrum. The $Co_A$ enzyme so isolated contained 1.0 ± 0.1 mol of Co(II) and a negligible amount (≤0.1 mol) of Zn(II). Note: For bands above 425 nm $\epsilon$ calculated per mole of RPase corresponds to $\epsilon$ per one Co(II), since only one site gives rise to these bands. Below 400 nm, $\epsilon$ per mole of RPase has contributions from both sites (cf. Figure 5).

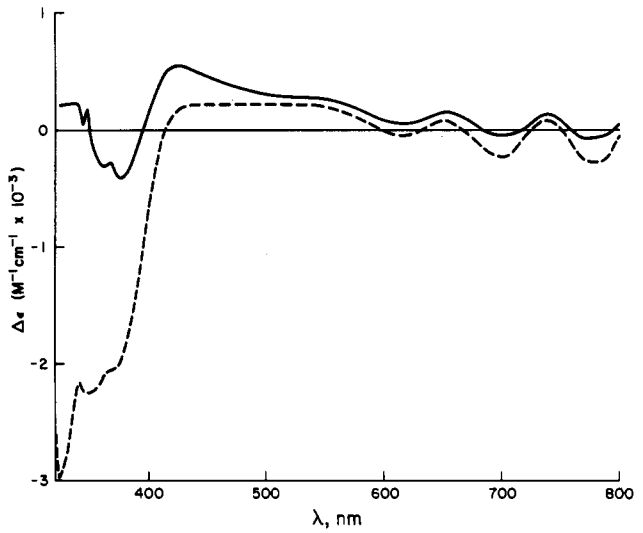


FIGURE 5: Reversible effect of PMPS on absorption spectrum of $Co_2$ RPase. Absorption spectra for $Co_2$ RPase (8.27 mg/mL) in TNG buffer were recorded under three conditions: (1) enzyme alone, (2) plus 35 equiv of PMPS, and (3) following the addition of DTT (0.1 mM to reverse the enzyme–PMPS complex; no EDTA was present in these buffers). (– –) $Co_2$ RPase–PMPS minus $Co_2$ RPase; (—) $Co_2$ RPase–PMPS + thiol minus $Co_2$ RPase. Atomic absorption analysis of the exhaustively dialyzed enzyme from this series of manipulations indicated 1.7 ± 0.2 mol of Co(II). Note that the small differences above 425 nm represent slight band shifts between the $Co_2$ RPase–PMPS complex and $Co_2$ RPase (see text).

and Methods. The data in Table VI show that $Co_2$ and $Cd_2$ enzymes were obtained with in vivo substitution efficiencies of ~100 and ~70–80%, respectively. A portion of each of these enzymes was treated with PMPS/EDTA under the conditions that yield a $Zn_1$ protein. Atomic absorption analysis of these enzymes reveal approximately 1 mol of Co(II) or Cd(II) in substituted enzymes (Table VI), providing further evidence for the specific placement of these metals into the original Zn(II) sites of the enzyme.[2]

Each $M_A$ enzyme (where M stands for metal) retained 82–108% of the activity in the starting $M_2$ enzyme on calf thymus DNA consistent with the results presented thus far. Similar relative activities were observed with the quantitative assay of Chamberlin et al. (1979) on T7 DNA. As pointed earlier, the specific activity of a particular preparation of RPase is characteristic of that preparation and is not directly comparable with another; however, all molecules able to synthesize RNA chains do so at a characteristic rate (17 $s^{-1}$ for the native *E. coli* enzyme) (Chamberlin et al., 1979). Thus, as a means to compare the specific transcriptional efficiencies of the various $M_A$ and $M_2$ enzymes, their rates of nucleotide incorporation into RNA chains (i.e., the rate of elongation) were determined. These are the pertinent values with which to compare the activities of various metallo RPases. As shown in Table VI, elongation rates ranging from 13 to 22 $s^{-1}$ were obtained; note that $Zn_A \approx Zn_2 = 17\ s^{-1}$, $Co_A \approx Co_2 = 21\ s^{-1}$, and $Cd_A \approx Cd_2 \approx 13\ s^{-1}$.

*Visible Absorption Spectroscopy of $Co_2$ and $Co_A$ RPases.* Substitution of Co(II) into the intrinsic Zn(II) sites of RPase provides a useful spectroscopic probe of the geometric arrangement about the metal ions as well as being indicative of the ligand donor atoms in the case of sulfur. Figure 4 presents the visible absorption spectra associated with the $Co_2$ enzyme. Three discrete d–d electronic transitions positioned at 760 ($\epsilon$ = 800), 710 ($\epsilon$ = 900), and 602 nm ($\epsilon$ = 1500) and a broad intense absorption band centered at about 484 nm ($\epsilon$ = 4000) are present. The first three transitions correspond approximately to those observed by Speckhard et al. (1977) at ~750, 703, and 584 nm for $Co_2$ RPase, although our measured molar extinction coefficients are considerably greater (see Discussion). In the region between 350 and 400 nm, several transitions appear with significantly greater $\epsilon$ values: 370 ($\epsilon$ = 9600) and 350 nm ($\epsilon$ = 9500). These transitions most likely arise from sulfur to Co(II) charge transfer, thus suggesting the presence of cysteine ligands to one or both intrinsic Co(II) ions. Figure 4 also gives a typical spectrum exhibited by the $Co_A$ enzyme formed by treatment of the $Co_2$ enzyme with PMPS/EDTA, subsequent reversal with thiol, and exhaustive dialysis. As can be seen, in the 400–800-nm region this enzyme shows almost identical intensities and minor band displacements relative to the $Co_2$ enzyme from which it originated. The major difference in this spectrum lies in the near-UV charge-transfer region; the intensities of the 370- and 350-nm transitions are reduced significantly. A qualitatively similar spectrum is obtained for the $Co_2$ RPase–PMPS complex, and if EDTA is present to form the $Co_1$ RPase on addition of DTT, there is no effect on the absorption spectrum (Figure 4).

[2] The metal contents and $^{115}Cd(II)$ exchange behavior characteristic of the biosynthesized enzyme obtained from the Cd(II)-supplemented cells are consistent with approximately 40–50% occupation of the A site with Zn(II) with 100% Cd(II) at the B site, i.e., a 40/60 mixture of $Zn_ACd_B$ and $Cd_ACd_B$ enzymes. Upon PMPS/EDTA treatment, the B-site Cd(II) is removed, and a 40/60 mixture of $Zn_A$ and $Cd_A$ enzyme results. Consistent with this interpretation is the rather facile exchange of ~1 g at Cd(II) with $^{115}Cd(II)$, the B-site Cd(II) (Table V). This partial occupation of the A site with Zn(II) was not observed in biosynthesized Co(II)-RPase.

Recall that if the PMPS–enzyme complex is reversed in the absence of EDTA, a $M_2$ species is formed (Table I). Figure 5 presents the spectral evidence for the re-formation of at least one M–S bond as Co(II) rebinds to the B site. The dashed curve represents a difference trace of the $Co_2$ RPase–PMPS complex less that of the starting $Co_2$ enzyme. Note again, relatively minor alterations associated with the 400–800-nm region with a large negative absorption band centered about 370 nm. The solid curve indicates the difference spectrum resulting when the $Co_2$ RPase–PMPS complex is reversed with thiol without chelator present, a reversal that also restores most of the minor band shifts above 500 nm. Ninety percent of the near-UV charge-transfer intensity is restored, suggesting the re-formation of the S–Co(II) bond(s) displaced by the mercurial. Atomic absorption analysis shows 1.7 mol of nondialyzable Co(II) in this enzyme species.

## Discussion

As a basis for understanding the regulation of transcription, a comprehensive knowledge of the relationship between structure and function of RNA polymerases must be attained. Prokaryotic RPases are multisubunit Zn(II) metalloenzymes and are largely homologous in subunit organization ($\alpha_2\beta\beta'\sigma$) among organisms. The enzyme from *E. coli* has emerged as the prototype bacterial RPase, since the majority of the detailed mechanistic studies have been carried out with this enzyme. The enzyme from *Bacillus subtilis* appears comparable in subunit composition and zinc content (Halling et al., 1977).

The recent demonstration that the single-chain RPase encoded by bacteriophage T7 contains no intrinsic Zn(II) (King et al., 1986) and transcribes DNA at an absolute rate 5–10 times that of most bacterial enzymes shows that Zn(II) does not play an obligatory role in transcription mechanisms. Thus, the role played by each of the two intrinsic Zn(II) ions in *E. coli* RPase in a more complex and controllable promoter-recognition sequence requires further definition. Previous studies employing complete denaturation and reconstitution of $M_1$ and $M_2$ RPases with various recoveries of activity have suggested catalytic, regulatory, and structural roles for either or both Zn(II) ions (Chatterji & Wu, 1982a,b; Chatterji et al., 1984; Solaiman & Wu, 1984, 1985). The ability to remove one of the intrinsic Zn(II) ions differentially by the PMPS/EDTA method offers a new opportunity to study the structure and function of the two sites independently under nondenaturing conditions.

Examination of the role of Zn(II) in other Zn(II) metalloenzymes of defined crystallographic structure including carbonic anhydrase, carboxypeptidase, alkaline phosphatase, alcohol dehydrogenase, and aspartate transcarbamoylase reveals that in some instances Zn(II) acts catalytically in the formation of mechanistic intermediates, while in others Zn(II) is involved in the stabilization of tertiary and/or quaternary structure. Aspartate transcarbamoylase (ATCase) provides an example where Zn(II) plays an exclusively structural role in the maintenance of the interface between catalytic (c) and regulatory (r) chains of the protein ($c_6r_6$) (Monaco et al., 1978). In this enzyme, all four sulfhydryl groups of each of the six r chains are involved in coordinating each of the six Zn(II) ions. Hunt et al. (1984) showed that treatment of ATCase with successive equivalents of the mercurial *p*-(hydroxymercuri)benzenesulfonate (PMPS) resulted in the linear release of Zn(II) ions from the protein until all 24 r chain sulfhydryls were titrated and the r and c chains had dissociated. Addition of thiol sufficient to reverse the mercurial reaction gave rise to reassociated protein of intact quaternary structure concomitant with the rebinding of Zn(II) (Hunt et al., 1984, 1985).

The adaption of PMPS to zinc removal from *E. coli* RPase was an attempt to avoid the denaturation and refolding steps that are drawbacks of previous methods to remove or replace the intrinsic zinc ions. In the case of RPase, the fact that addition of 10–16 equiv of PMPS removes $Zn_B$, but also inhibits transcriptional activity (Figure 1), suggests that two classes of –SH groups are involved initially, since the PMPS–EDTA–thiol reversal restores activity, but not $Zn_B$ (Figure 2). A recent paper by Chertov et al. (1985) suggests that organic mercurials inhibit RNA elongation by preventing the ability of the enzyme to bind to promoter-containing DNA fragments. That we have observed largely similar binding capacities of $Zn_1(Zn_A)$ and $Zn_2$ enzymes on T7 DNA A1 and A2 promoter-containing *Hpa*II fragments (D. Giedroc and J. Coleman, unpublished observations) provides further support for the contention that removal of B-site Zn(II) occurs as a separate consequence of the reaction with the mercurial distinct from the coincident loss of enzyme activity, which is restored without rebinding of Zn(II) when thiol and EDTA are used (Figure 2).

Exchange experiments with $^{65}Zn(II)$ suggest that the presence of PMPS complexed to the Zn(II) enzyme does not significantly increase the ability of $^{65}Zn(II)$ to exchange with the B-site Zn(II) (Table V). One interpretation of these data is that the so-called enzyme-released Zn(II) actually remains associated with the enzyme through one or more non-sulfhydryl ligands following mercurial reaction such that diffusion of the Zn(II) into the $^{65}Zn(II)$ pool cannot occur readily. EDTA, however, must be able to chelate the Zn(II) entirely from the modified protein molecule whereas it is unable to do so in the native state. Once all of the reagents have been removed from $Zn_A$ RPase, it has proved difficult to rebind metal ions of any type to the B site, suggesting that some local conformational perturbations may have occurred at the metal-free B site.

In contrast, exchange experiments with $^{115}Cd$ [or nonradioactive Cd(II)] show that excess Cd(II) presented to either the Zn enzyme–PMPS complex or the native $Zn_2$ enzyme in the absence of EDTA gives rise, in both cases, to the formation of a mixed-metal hydrid $Zn_ACd_B$ enzyme (Tables I and V). Therefore, Cd(II) alone in sufficient quantities seems able to displace the $Zn_B$. The presence of one or more cysteinyl ligands to the B-site Zn(II) is compatible with these findings given the greater ability of Cd(II) as well as the organic mercurial, relative to $^{65}Zn(II)$, to displace this Zn(II) ion (Table V).

No significant change in the ability of the native enzyme to bind to and transcribe RNA from $\sigma$-requiring promoter sequences results from removal of B-site Zn(II). Since the $Zn_A$ RPase shows little capacity to bind Zn(II) at the B site, even in the presence of thiols (Table V), it seems unlikely that Zn(II) rebinds to site B during the assay. To eliminate this possibility as far as possible, the assay of the $Zn_A$ enzyme was run in 1–2 mM EDTA without any effect on the rate of RNA synthesis. Consideration of the facile binding of Zn(II) in RNA polymerase assays has recently been discussed in detail (King et al., 1986). $Zn_A$ and $Zn_2$ holoRPases have similar rates of elongation along T7 DNA (Table VI). In the latter measurements, we have isolated the specific transcripts by radioautography of the RNA gels and quantitated the amount of mRNA by counting. Since the transcript lengths are identical between $Zn_A$ and $Zn_2$ RPases, the absence of $Zn_B$ does not effect $\sigma$-aided recognition and initiation, the elongation rate, or recognition of the specific terminator at 18.8%

of the T7 genome. The similarity of the secondary structure of the native, $Zn_A$, and $Zn_ACd_B$ preparations is shown by UV circular dichroism spectroscopy (Table IV).

The possible function of this "PMPS-removable" B-site Zn(II) in RPase function cannot be directly ascertained from the present experiments. Aside from the obvious interpretation that this ion cannot be mechanistically crucial for RNA synthesis, B-site Zn(II) perhaps plays an accessory role in maintenance of structure. We do not dismiss $Zn_B$ from playing any role in transcription, since it is entirely possible that transcription from promoters regulated by CAP or other transcription factors may be significantly altered by the loss of $Zn_B$. Studies of such regulated promoters are presently under way.

Since the basic polymerization reaction appears unaffected by removal of B-site Zn(II), the function of the A-site Zn(II) can be probed directly. Specifically, Wu and Wu and coworkers through a variety of denaturation–reconstitution techniques have suggested that at least one of the two intrinsic M(II) ions is catalytically critical, since the nucleotide base of an initiating nucleoside triphosphate appears to be close to the Co(II) ion in a reconstituted $Co_1Zn_1$ hybrid enzyme (Chatterji & Wu, 1982b; Chatterji et al., 1984).

Visible absorption spectroscopy of $Co_2$ and $Co_A$ RPases show that the visible "d–d" Co(II) absorption bands are associated only with the A site (Figures 4 and 5). The positions and magnitudes of the d–d transitions of the $Co_A$ enzyme are consistent with this Co(II) occupying a tetrahedral or distorted tetrahedral environment. In contrast, the B-site Co(II) is essentially spectrally silent in the visible d–d transition region and therefore is possibly in an octahedral coordination geometry, perhaps with solvent access to at least one site. Both A and B sites have strong charge-transfer bands in the near ultraviolet, which suggests that both sites involve sulfur ligation. The visible absorption bands have much larger extinction coefficients than previously reported (Speckhard et al., 1977). This is partly due of course to assignment to a single Co(II) site, but also to larger experimental absorption in our samples.

In the case of A-site Co(II), the presence of two transitions in the 700–800-nm range accompanied by a somewhat weak and much broader maximum at about 600 nm and intense charge-transfer bands below 400 nm suggests a tetrahedral, tetrathiolate coordination about A-site Co(II). Both band energies and intensities are similar to those shown by Co(II) in other tetrathiolate coordination sites. The spectrum closely matches those of Co(II)-substituted aspartate transcarbamoylase (Johnson & Schachman, 1983), alcohol dehydrogenase (structural site) (Maret et al., 1979), metallothionein (Vasak et al., 1981), and rubridoxin (May & Kuo, 1978). All of these are known by crystallographic methods to have four thiolate ligands to each Zn(II). These Co(II) protein complexes and the model compound tetrathio(phenolato)cobaltate(II) (Swenson et al., 1978) all exhibit two transitions in the 700–800-nm range: one at 740–760 nm and another at 660–700 nm, as well as the near-UV charge-transfer bands. The presence of the charge transfer also appears associated with relatively more intense visible transitions than found in the mixed N,O tetrahedral Co(II) sites. Sulfhydryl groups have recently been implicated in metal binding by RPase, since potassium tetrathionate blocks reassociation of metal ions during reconstruction of urea-denatured apoRPase (Solaiman & Wu, 1985).

A tetrathiolate coordination complex is electron-rich, which tends to restrict either expansion of the coordination sphere by electron-donating ligands or solvent access to the Zn(II) ion. Thus, if site A plays a direct role in catalysis, it may not do it by coordinating a substrate or template ligand but rather by providing essential conformational constraints. In this connection, we have not been able to observe consistent changes in the $Co_A$ RPase absorption spectrum by adding MgATP. While slight shifts in the position of the visible maxima occurred in some preparations when very high sensitivity difference spectra were recorded directly, we are reluctant as yet to interpret these minor changes. A-site Zn(II) is very tightly incorporated into the RPase structure, since no chelating agent or sufhydryl reagent that we have tested has been able to remove A-site Zn(II). This site may be associated with $\beta'$.[3] We have found that relatively low concentrations of urea (2.5 M), which do not normally labilize the A-site Zn(II), allow the PMPS/EDTA mixture to remove A-site Zn(II), producing an inactive apoRPase (D. Giedroc and J. Coleman, unpublished results). Previous studies have all required 6 M urea to remove all the Zn(II) (Wu et al., 1977).

In conclusion the gentle removal of one of the two Zn(II) ions in *E. coli* RPase by PMPS/EDTA treatment under nondenaturing conditions has allowed the separation of the functional and structural features of the two intrinsic Zn(II) sites. The different absorption spectra when these two sites are occupied by Co(II) establish one (site A) as tetrahedral, probably with four thiolate ligands. The other (site B) is easily removed by PMPS/EDTA and has no significant d–d absorption bands when Co(II) is substituted and is apparently some variant of octahedral geometry. The metal ion in the site B has no detectable effect on any of the steps in transcription including $\sigma$-dependent promoter recognition.

**Registry No.** Zn, 7440-66-6; DNA-dependent RNA polymerase, 9014-24-8.

## REFERENCES


Allison, L. A., Mayle, M., Shales, M., & Engles, C. J. (1985) *Cell (Cambridge, Mass.) 42*, 599–610.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Brändén, C.-I., Jörnvall, H., Eklund, H., & Furugren, B. (1975) *Enzymes (3rd Ed.) 11*, 103–190.

Burgess, R. R. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 69–100, Cold Spring Harbor Press, Cold Spring Harbor, NY.

Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634–4638.

Chamberlin, M. J. (1974) *Annu. Rev. Biochem. 43*, 721–775.

Chamberlin, M. J., Nierman, W. C., Wiggs, J., & Neff, N. (1979) *J. Biol. Chem. 254*, 10061–10069.

Chamberlin, M. J., Kingston, R., Gilman, M., Wiggs, J., & deVera, A. (1983) *Methods Enzymol. 101*, 540–568.

Chatterji, D., & Wu, F. Y.-H. (1982a) *Biochemistry 21*, 4651–4656.

Chatterji, D., & Wu, F. Y.-H. (1982b) *Biochemistry 21*, 4657–4664.


[3] The N-terminal region of $\beta'$ contains a Cys-rich sequence between residues 58 and 88, Cys-$X_{11}$-Cys-X-X-Cys-$X_{12}$-Cys-X-X-Cys-, which has a distribution not unlike the Cys distribution occurring in known Zn enzymes with tetrathiolate coordination sites. Such a cluster occurs nowhere else in the sequence of $\beta'$ (Ovchinnikov et al., 1982). Proof that this constitutes a zinc site will require overproduction of a "native" folded $\beta'$ subunit and demonstration of an isolated Zn(II) binding site. It has recently been shown that the largest subunits of yeast RNA polymerases II and III contain many regions of amino acid sequence homologous to sequences in $\beta'$ of *E. coli* (Allison et al., 1985). Like $\beta'$, both of these eukaryotic RNA polymerase subunits contain nearly identical Cys-X-X-Cys-$X_6$-Cys···Cys-X-X-Cys clusters at their N-terminal ends and contain no other clustered Cys residues.

Chatterji, D., Wu, C.-W., & Wu, F. Y.-H. (1984) *J. Biol. Chem. 259*, 284–289.
Chertov, O. I., Selivchenko, O. A., & Lipkin, V. M. (1985) *Bioorg. Khim. 11*, 492–498.
Coleman, J. E. (1974) *Biochem. Biophys. Res. Commun. 60*, 641–648.
Coleman, J. E., & Vallee, B. L. (1960) *J. Biol. Chem. 235*, 390–395.
Davison, B. L., Leighton, T., & Rabinowitz, J. C. (1979) *J. Biol. Chem. 254*, 9220–9226.
Drum, D. E., & Vallee, B. L. (1970) *Biochemistry 9*, 4078–4086.
Golomb, M., & Chamberlin, M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 760–764.
Gonzalez, N., Wiggs, J., & Chamberlin, M. J. (1977) *Arch. Biochem. Biophys. 182*, 404–408.
Halling, S. M., Sanchez-Anzaldo, F. J., Fukuda, R., Doi, R. H., & Meares, C. F. (1977) *Biochemistry 16*, 2880–2884.
Hanna, M. M., & Meares, C. F. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4238–4242.
Hanna, M. M., & Meares, C. F. (1983b) *Biochemistry 22*, 3546–3551.
Harding, J. D., & Beychok, S. (1973) *Biochem. Biophys. Res. Commun. 51*, 711–717.
Hillel, Z., & Wu, C.-W. (1977) *Biochemistry 16*, 3334–3342.
Hunt, J. B., Neece, S. H., Schachman, H. K., & Ginsberg, A. (1984) *J. Biol. Chem. 259*, 14793–14803.
Hunt, J. B., Neece, S. H., & Ginsberg, A. (1985) *Anal. Biochem. 146*, 150–157.
Ishihama, A. (1972) *Biochemistry 11*, 1250–1258.
Iwakura, Y., Ishihama, A., & Yuru, T. (1973) *Mol. Gen. Genet. 133*, 1–23.
Johnson, R. S., & Schachman, H. K. (1983) *J. Biol. Chem. 258*, 3528–3538.
King, G. C., Martin, C. T., Pham, T. T., & Coleman, J. E. (1986) *Biochemistry 25*, 36–40.
Levine, B. J., Orphenos, P. D., Fischmann, B. S., & Beychok, S. (1980) *Biochemistry 19*, 4808–4814.
Loew, P., Hager, D., & Burgess, R. R. (1979) *Biochemistry 18*, 1344–1352.
Maret, W., Andersson, I., Dietrich, H., Schneider-Bernlhr, H., Einarsson, R., & Zeppezauer, M. (1979) *Eur. J. Biochem. 98*, 501–512.
May, S., & Kuo, J. Y. (1978) *Biochemistry 17*, 3333–3338.
Mildvan, A. S., & Loeb, L. A. (1979) *CRC Crit. Rev. Biochem. 6*, 219–244.
Miller, J. A., Serio, G. F., Howard, R. A., Bear, J. L., Evins, J. E., & Kimbell, A. P. (1979) *Biochim. Biophys. Acta 579*, 291–297.
Monaco, H. L., Crawford, J. L., & Lipscomb, W. N. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5276–5280.
Ovchinnikov, Y. A., Monastyrskaya, G. S., Gubanov, V. V., Guryev, S. O., Salomatina, I. S., Shuvaeva, T. M., Lipkin, V. M., & Sverdlov, E. D. (1982) *Nucleic Acids Res. 10*, 4035–4044.
Park, C. S., Hillel, Z., & Wu, C.-W. (1980) *Nucleic Acids Res. 8*, 5895–5912.
Rabussay, D., & Zillig, W. (1969) *FEBS Lett. 5*, 104–106.
Solaiman, D., & Wu, F. Y.-H. (1984) *Biochemistry 23*, 6369–6377.
Solaiman, D., & Wu, F. Y.-H. (1985) *Biochemistry 24*, 5077–5083.
Speckhard, D. C., Wu, F. Y.-H., & Wu, C.-W. (1977) *Biochemistry 16*, 5228–5234.
Swenson, D., Baenziger, N. C., & Coucouvanis, D. (1978) *J. Am. Chem. Soc. 100*, 1932–1934.
Vasak, M., Kägi, J. H. R., Holmquist, B., & Vallee, B. L. (1981) *Biochemistry 20*, 6659–6664.
von Hippel, P. H., Bear, D. G., Morgan, W. D., & McSwiggen, J. A. (1984) *Annu. Rev. Biochem. 53*, 389–446.
Walton, K. E., FitzGerald, P. C., Herrman, M. S., & Behnke, W. D. (1982) *Biochem. Biophys. Res. Commun. 108*, 1353–1361.
Wu, C.-W., Wu, F. Y.-H., & Speckhard, D. C. (1977) *Biochemistry 16*, 5449–5454.
Yarbrough, L. R., & Hurwitz, J. (1974) *J. Biol. Chem. 249*, 5400–5404.
Yarbrough, L. R., & Wu, C.-W. (1974) *J. Biol. Chem. 249*, 4079–4085.
Yura, T., & Ishihama, A. (1979) *Annu. Rev. Genet. 13*, 59–97.
Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A., & Seifert, W. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35*, 47–58.
Zillig, W., Palm, P., & Heil, A. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 101–125, Cold Spring Harbor Press, Cold Spring Harbor, NY.