# Preparation and Characterization of Various *Escherichia coli* RNA Polymerases Containing One or Two Intrinsic Metal Ions†

Daniel Solaiman and Felicia Ying-Hsiueh Wu*

*Department of Pharmacological Sciences, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794*

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ABSTRACT: The *Escherichia coli* DNA-dependent RNA polymerase (RPase) holoenzyme ($\alpha_2\beta\beta'\sigma$) possesses 2 mol equiv of Zn: $\beta$ and $\beta'$ subunits each contain one Zn ion. An in vitro metal-substitution method developed earlier (method I) was used to remove the two intrinsic Zn ions and then to reconstitute other metal ions into the $\beta$ subunit of RPase. One Cd or Hg ion was successfully reconstituted into half-active enzymes (rec-$Cd_1$- or rec-$Hg_1$-RPase), while Mn or Ni ion was not incorporated. A new, simplified in vitro metal-substitution method (method II), which omitted the low-pH treatment and subsequent urea dialysis in method I, was devised in this study. Consequently, Zn or Cd could be incorporated into both the $\beta$ and $\beta'$ subunits, resulting in rec-$Zn_2$- or rec-$Cd_2$-RPase, respectively. However, only one Hg was incorporated, probably due to steric hindrance by the large size of the Hg ion, while Mn, Ni, or Cr was not bound by the reconstituted enzyme, which instead incorporated only one Zn. Analysis of the metal content of various reconstituted RPases indicated that without low-pH treatment Zn bound to both the $\beta$ and $\beta'$ subunits when Zn concentrations were higher than $2 \times 10^{-6}$ M, but it bound only to the $\beta'$ subunit at lower concentrations. Moreover, low-pH treatment destroys the metal binding site in the $\beta'$ subunit. The metal sites on the $\beta$ and $\beta'$ subunits did not have significant affinity for the transition metals such as Mn, Ni, and Cr. However, all group 12 metals (Zn, Cd, and Hg) bound tightly to the enzyme, yielding reconstituted RPases with proper conformation (13*S*) as shown by the density gradient sedimentation and fluorescence spectroscopic analysis. The activities of these Cd or Hg RPases in binding pAR1435 plasmid DNA and catalyzing the abortive initiation reactions at the A1 promoter of T7 DNA paralleled their activities in total RNA synthesis. The favorable binding of Cd and Hg to RPase suggested that sulfhydryl groups of RPase may be involved in the metal binding. This notion was supported by the results obtained from sulfhydryl blocking experiments using $K_2S_4O_6$.

Many nucleotidyl transferases including DNA and RNA polymerases from both procaryotic and eucaryotic sources have been shown to be Zn metalloenzymes (Mildvan & Loeb, 1979; Wu & Wu, 1981, 1983). The *Escherichia coli* DNA-dependent RNA polymerase (RPase),[1] a 495 000-dalton oligomeric ($\alpha_2\beta\beta'\sigma$) enzyme, was first shown to contain two tightly bound Zn ions by Scrutton et al. (1971). Subunits $\beta$ and $\beta'$ each contain one Zn ion (Wu et al., 1977; Miller et al., 1979). We have recently succeeded in removing the two intrinsic Zn ions from RPase to obtain inactive apoenzyme and shown that reconstitution of an active enzyme can occur only in the presence of exogenous Zn ions (Solaiman & Wu, 1984). These results show directly for the first time that Zn is an indispensable component of the active RPase playing both structural and catalytic roles.

Although the importance of intrinsic Zn in RPase is now established, the exact role played by the metal is still poorly understood. Information regarding the role of intrinsic metal in RPase largely came from our earlier studies using paramagnetic metal ion substituted enzymes obtained by in vivo (Speckhard et al., 1977; Wu et al., 1977) and in vitro (Chatterji & Wu, 1982a,b; Chatterji et al., 1984) metal substitution. With such reconstituted RPases, biophysical studies become feasible since the paramagnetic metal substituents, unlike Zn ions, possess measurable optical and magnetic properties. By in vivo metal substitution, we obtained Co–Co RPase with enzymatic activity similar to that of native Zn–Zn RPase. Zn–Zn and Co–Co RPases are very similar biochemically and physically except for their efficiencies in recognizing promoters and initiating RNA chains. Furthermore, the visible absorption spectrum of the Co–Co RPase was

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[1] Abbreviations: RPase, RNA polymerase; Zn–Zn (or $Zn_2$) or Co–Co RPase, RNA polymerase containing two intrinsic Zn or Co ions, respectively; Co–Zn, Mn–Zn, Ni–Zn, or Cu–Zn RPase, RNA polymerase containing one intrinsic Zn ion and one intrinsic Co, Mn, Ni, or Cu ion, respectively; apo-RPase, RNA polymerase containing no intrinsic metal ions; rec-RPase and rec-RPase′, RNA polymerase reconstituted by methods I and II, respectively, as described under Materials and Methods; rec-$M_1$-RPase or rec-$M_1$-RPase′, reconstituted RPase containing one metal (M); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; $\beta$-ME, $\beta$-mercaptoethanol; $K_2S_4O_6$, potassium tetrathionate; NMR, nuclear magnetic resonance; bp, base pair. The contents of the various buffers were as follows: A, 50 mM Tris-HCl (pH 8.0), 20 mM $\beta$-ME, 10 mM EDTA, and 8 M urea; B, 50 mM KCl, 2 mM $\beta$-ME, and 10 mM EDTA (pH 2.2); C, buffer A without 10 mM EDTA; D, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.5 M KCl, 2 mM $\beta$-ME, and 20 glycerol; E, 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, and 1 M KCl; F, 10 mM Tris-HCl (pH 7.9), 0.5 M KCl, 5 mM $\beta$-ME, and 0.5 mM EDTA; G, 50 mM Tris-HCl (pH 8.0), 0.5 M KCl, 1 mM EDTA, 20% (v/v) glycerol, 8 M urea, and 10 mM $K_2S_4O_6$; H, buffer D without 2 mM $\beta$-ME; I, buffer G containing 10 mM DTT instead of 10 mM $K_2S_4O_6$; J, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.1 mM DTT, 0.1 M KCl, and 5% glycerol.

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perturbed upon addition of nucleoside triphosphate or template analogue, suggesting that the metals may be involved in substrate and template binding. We have subsequently developed an in vitro method to selectively substitute the intrinsic metal in the $\beta$ subunit with other divalent metal ions yielding metal hybrid RPases (e.g., Co–Zn, Mn–Zn, Ni–Zn, and Cu–Zn RPases) with different enzyme activities (Chatterji & Wu, 1982a). NMR studies with the Co–Zn and Mn–Zn RPases in the absence and presence of DNA template revealed that the substituted metal in the $\beta$ subunit is located at the initiation nucleotide binding site and is in direct coordination with the base moiety of the substrate (Chatterji & Wu, 1982b; Chatterji et al., 1984). Thus, the metal in the $\beta$ subunit plays a regulatory role in the recognition and orientation of the initiation nucleotide for enzyme catalysis.

We now report on our continuing studies concerning the role of intrinsic metal in RPase. Using the recently developed in vitro metal-substitution method (Solaiman & Wu, 1984) and its simplified version as presented here, we have prepared a variety of metal-substituted RPases in which the intrinsic Zn ions were first completely removed and replaced by various divalent metal ions. The biochemical and biophysical properties of the reconstituted RPases were then investigated. Results from this study also yielded information regarding the nature of the metal binding sites of RPase.

## Materials and Methods

*Materials.* Unlabeled nucleoside triphosphates (NTP) were obtained from P-L Biochemicals; $^{32}$P- and $^{3}$H-labeled NTP were from either ICN Pharmaceuticals or New England Nuclear. Ultrapure urea and Tris were purchased from Schwarz/Mann. Calf thymus DNA (type I, highly polymerized) and EDTA were products of Sigma. Potassium tetrathionate was obtained from K&K Laboratories Inc., and nitrocellulose filters (BA 85) were from Schleicher & Schuell. Metal salts were purchased from Baker Chemical Co. All metal standard solutions for atomic absorption analysis were products of Fisher Co. All other biochemicals were of highest purity obtained commercially.

*Preparation of RPase.* Enzyme was purified from *E. coli* MRE 600 cells (Grain Processing) according to the method of Burgess & Jendrisak (1975). Further purification and determination of enzyme concentration were performed as described earlier (Solaiman & Wu, 1984). RPase enzyme activity was assayed according to the procedure of Wu & Wu (1973). One unit of enzyme activity is defined as 1 nmol of $^{3}$H-labeled nucleoside monophosphate incorporated into the acid-insoluble material in 20 min.

*Preparation of Apo-RPase and Metal-Substituted RPases (Rec-RPases and Rec-RPase's).* Two protocols were employed to reconstitute *E. coli* RPase in the absence or presence of various exogenous metal ions. Method I had been described previously (Solaiman & Wu, 1984), and the reconstituted enzymes were designated as rec-RPases. Method II is the simplified protocol derived from method I. In method II, 10 mg of RPase (0.7 mL) was dialyzed against 250 mL of buffer A at room temperature for 2~2.5 h. The dialyzed enzyme was diluted 5-fold with buffer D containing $5 \times 10^{-5}$ M of the desired metal salt and dialyzed against 500 mL of the same buffer D with metal ions for 3.5~4.5 h at room temperature and then overnight at 4 °C. The reconstituted enzymes (designated as rec-RPase's) were concentrated to about 1 mg/mL by ammonium sulfate precipitation and stored for further use. For isolation of the (13*S*)-rec-RPase species (Solaiman & Wu, 1984), the renatured enzyme was further purified by centrifugation on 10–30% (v/v) glycerol density gradient as described below. The (13*S*)-RPase species represents the fraction of the rec-RPase that is active and sediments similarly to the native RPase (solaiman & Wu, 1984).

*Density Gradient Ultracentrifugation.* Glycerol gradient sedimentation was performed as described (Burgess & Travers, 1971). Enzyme was dialyzed at 4 °C for at least 4 h against buffer E. Dialyzed enzyme was layered onto 25~26 mL of 10–30% (v/v) gradient in buffer E and centrifuged in a Beckman SW 25 rotor at 21 000 rpm for 42 h at 4 °C. Sucrose density gradient sedimentation analysis was carried out by the method of Martin & Ames (1961). After dialysis against buffer F for >4 h at 4 °C, the enzyme was layered onto 4.6 mL of 5–20% (w/v) gradient in buffer F and sedimented in a Beckman SW 65 rotor at 38000*g* for 17 h at 4 °C. Fractions (0.25–0.75 mL) were collected from the top of the gradient. Protein concentration, metal content, and enzyme activity of each fraction were measured as described under Materials and Methods. Peak fractions of interest were pooled and concentrated for further analysis. The percent recovery of activity and protein was 20–40% and 60–90%, respectively.

*Preparation of Plasmid DNAs.* Plasmid pAR1435 (a gift from Drs. W. Studier and A. H. Rosenberg, Biology Department, Brookhaven National Laboratory, Upton, NY) is a pBR322 derivative containing a 102-bp fragment of T7 DNA cloned at the *Bam*HI site. Since the cloned T7 DNA fragment includes the A1 promoter region, the plasmid is suitable for use as a template in the abortive initiation assay. The plasmid DNAs with and without $^{32}$P label were prepared as described earlier (Solaiman & Wu, 1984).

*Abortive Initiation Assay.* The procedure of Oen et al. (1979) was employed, except plasmid DNA (pAR1435) was used as a template (Solaiman & Wu, 1984).

*Nitrocellulose Filter Binding Assay.* The binding assay was performed as described by Hinkle & Chamberlin (1972) except $^{32}$P-labeled pAR1435 DNA was used (Solaiman & Wu, 1984).

*Blockage and Regeneration of Sulfhydryl Groups in RPase.* The procedures described previously (Yarbrough & Wu, 1974; Wu et al., 1977) were used to block the SH groups of the denatured apo-RPase [denatured RPase 2, Scheme I of Solaiman & Wu (1984)]. RPase (1.2 mg) in 1.25 mL of solution B (without 10 mM EDTA) was dialyzed against 250 mL of buffer G sequentially for 2 h at room temperature, overnight in the cold room, and then 1 h at room temperature. Reconstitution of the tetrathionate-blocked RPase was attempted by dialysis against buffer H in the presence of $10^{-4}$ M $CdCl_2$. The control sample was prepared similarly, except that after overnight dialysis in the cold room the tetrathionate-treated enzyme was dialyzed against buffer I for 1 h at room temperature. In a separate experiment, 2.3 mg of native RPase (7.7 mg/mL) was dialyzed against 250 mL buffer A without $\beta$-ME for 2 h at room temperature. $N_2$ was bubbled through the buffer during dialysis to maintain a reducing atmosphere since $\beta$-ME had been omitted in buffer A to avoid subsequent reaction with $K_2S_4O_6$. The denatured enzyme was incubated with $10^{-2}$ M $K_2S_4O_6$ at room temperature for 30 min. Excess tetrathionate was removed by dialysis against three changes of buffer A without $\beta$-ME under $N_2$ atmosphere. Half of the tetrathionate-treated enzyme was incubated with $10^{-2}$ M DTT at room temperature for 1 h in an attempt to free the $S_2O_3$-blocked groups. Both aliquots of enzyme with free and blocked SH groups were dialyzed against buffer C containing $5 \times 10^{-5}$ M $CdCl_2$ for 3.5–4.5 h at room temperature and overnight in the cold room. Enzyme activity and metal content

Table I: Metal Content and Relative Enzyme Activities of Various Reconstituted RPases

| exogenous metal[a] | metal content (mol/mol of enzyme)[b] Zn | other metal[c] | relative enzyme activity (%)[d] |
|---|---|---|---|
| | (A) Rec-RPases[e] | | |
| *f* | 0.2 ± 0.1[g] | | 3[g] |
| Zn(II) | 1.0 ± 0.2[g] | | 49[g] |
| Cd(II) | 0.2 ± 0.1 | 1.2 ± 0.1 | 51 |
| Hg(II) | 0.1 ± 0.1 | 1.3 ± 0.1 | 72 |
| Mn(II) | 0.6 ± 0.1 | 0.1 ± 0.1 | 20 |
| Ni(II) | 0.4 ± 0.2 | 0.7 ± 0.1 | 15 |
| | (B) Rec-RPase's[h] | | |
| *f* | 1.1 ± 0.1 | | 57 |
| Zn(II) | 2.1 ± 0.1 | | 94 |
| Cd(II) | 0.2 ± 0.1 | 1.6 ± 0.2 | 89 |
| Hg(II) | 0.3 ± 0.1 | 1.0 ± 0.3 | 72 |
| Co(II) | 0.3 ± 0.1 | 1.1 ± 0.1 | 4 |
| Mn(II) | 1.2 ± 0.1 | 0 | 69 |
| Ni(II) | 1.1 ± 0.1 | 0.1 ± 0.1 | 55 |
| Cr(III) | 0.9 ± 0.1 | 0.2 ± 0.1 | 47 |

[a] Exogenous metal ions as chloride salts were added in the reconstitution steps of method I (Solaiman & Wu, 1984). [b] Determined by atomic absorption spectrometry. [c] The exogenous metal being incorporated into RPase. [d] Expressed as the percent of enzyme activity of native RPase. [e] Rec-RPases were obtained by method I as described (Solaiman & Wu, 1984). [f] No exogenous metal added. However, atomic absorption analysis showed that $2 \times 10^{-6}$ M residual Zn was present in the buffer. [g] Data were taken from our previous paper (Solaiman & Wu, 1984). [h] Rec-RPase's were obtained by method II as described under Materials and Methods and further purified as 13*S* species by glycerol gradient sedimentation.

of the reconstituted enzymes were measured as described under Materials and Methods.

*Metal Determination.* Metal content was determined by using a Perkin-Elmer Model 4000 atomic absorption spectrophotometer equipped with a HGA 400 graphite furnace. All labware that came in contact with the samples was soaked overnight in 30% $HNO_3$ or 10 mM EDTA and rinsed extensively with deionized, distilled water (DDW) before use. Prior to measurement, protein (0.1–0.5 mg/mL) was dialyzed at least 16 h at 4 °C against buffer J. Before Zn analysis, protein was diluted with DDW to a concentration of 10–50 μg/mL, and 10 μL of solution was introduced into the graphite furnace for each determination. Zn content was calcultaed from the standard curve constructed by using Zn standard solution with DDW and buffer J as diluents. Native RPase was used as a positive control in Zn determintions. Analyses for Co, Cd, Ni, Mn, and Cr were similarly performed except that samples were diluted with 1% (w/v) $HNO_3$ to yield a protein concentration of 250 μg/mL, and the wavelengths and conditions used were those suggested by Perkin-Elmer. Hg content was analyzed after the samples had been diluted with 6% HCl and 2% $H_2O_2$ as described (Alder & Hickman, 1977). For all metal determinations, duplicate or triplicate samples were analyzed.

## Results

*Preparation of Apo-RPases and Metal-Substituted RPases (Rec-RPases and Rec-RPase's).* *E. coli* RPase holoenzyme was first rendered metal free by sequential dialysis against buffer A and solution B by method I as described previously (Solaiman & Wu, 1984). The denatured, metal-free protein was reconstituted by dialysis against buffer C and then buffer D, without or with the addition of various metal ions in both buffers. Table IA shows the metal content and relative enzyme activities of the reconstituted RPases obtained in the absence or presence of various metal ions in buffers C and D. As reported earlier (Solaiman & Wu, 1984), when $ZnCl_2$ was either omitted or added in the reconstitution steps, the inactive apo-RPase or half-active (49%) rec-$Zn_1$-RPase was obtained, respectively. The addition of $CdCl_2$ or $HgCl_2$ in buffers C and D yielded rec-RPases with 51 ± 10% or 72 ± 10% enzyme activity, respectively, as compared to that of the native enzyme. These renatured RPases contained 1.2 ± 0.1 mol of Cd or 1.3 ± 0.1 mol of Hg per mole of enzyme and were designated as rec-$Cd_1$- or rec-$Hg_1$-RPase. Since the reconstituted apo-RPase in which the metal binding sites were not occupied lacked enzyme activity, it appeared that Cd or Hg ion had been incorporated respectively into rec-$Cd_1$-RPase or rec-$Hg_1$-RPase. The location of the unique metal in the reconstituted RPase had been shown to be in the β subunit (solaiman & Wu, 1984). Table IA also shows the results obtained when either $MnCl_2$ or $NiCl_2$ was added in the reconstitution buffers; the reconstituted enzymes exhibited very low enzyme activity (15~20%). The Mn content in the renatured Mn enzyme was negligible (0.1 ± 0.1 mol/mol of enzyme), but the Zn content was relatively high (0.6 ± 0.1 mol/mol of enzyme). Similarly, enzyme reconstituted with Ni contained 0.4 ± 0.2 mol of Zn and only 0.7 ± 0.1 mol of Ni per mole of enzyme. These results indicated that the binding of Ni and Mn ions by apo-RPase was less favorable than that of Cd, Hg, or Zn ions, which are group 12 metals.[2] Reconstitution using $CoCl_2$ or $CrCl_3$ has also been attempted, but results were not consistent due to the appearance of metal aggregates in buffer D (data not shown).

In an attempt to obtain reconstituted RPases containing two exogenously added metals, a modified method (method II) for the reconstitution of denatured, metal-free enzyme was developed. Method II differed from method I in that (1) the two dialysis steps against the low-pH solution B and subsequent buffer C containing urea (which were harsh conditions for RPase) were omitted, (2) lower metal concentrations ($10^{-5}$ M) were used in buffer D, and (3) the time of dialysis against buffer D was lengthened by 1 h. The denatured apo-RPase obtained by method II was inactive and did not contain Zn, as shown by atomic absorption analysis. Therefore, the metal found in the renatured RPase' was assumed to have originated from external sources and to have been incorporated into the enzyme during reconstitution. When method II was used to reconstitute RPase in the absence of external metal ion in reconstitution buffer D, we obtained enzyme that contained 1.1 ± 0.1 mol of Zn/mol of enzyme and had 57 ± 10% of the activity of native RPase (Table IB). Since buffer D contained $2 \times 10^{-6}$ M Zn even without addition of metal exogenously, the unique Zn ion in the rec-RPase' appeared to originate from the residual Zn contaminant. When $5 \times 10^{-5}$ M $ZnCl_2$ was added exogenously in buffer D, the reconstitution generated rec-RPase', which contained 2.1 ± 0.1 mol of Zn/mol of enzyme and was 94% active. These results seemed to show that the two metal binding sites in RPase differed in their affinity for Zn. When Zn concentration was low ($<2 \times 10^{-6}$ M), as in the case of reconstitution in the absence of metal addition, only the strong binding site was saturated with metal, while at high external Zn concentration (e.g., $5 \times 10^{-5}$ M) both strong and weak binding sites were occupied by the metal.

[2] In this paper the periodic group notation is in accord with recent actions by IUPAC and ACS nomenclature committees. A and B notation is eliminated because of wide confusion. Groups IA and IIA become groups 1 and 2. The d-transition elements comprise groups 3 through 12, and the p-block elements comprise groups 13 through 18. (Note that the former Roman number designation is preserved in the last digit of the new numbering: e.g., III → 3 and 13.)

Table IB shows the metal content and relative enzyme activities of the rec-RPase's obtained when various metal ions were added to buffer D. With $CdCl_2$ as an external metal source, renatured enzyme with 1.6 ± 0.2 mol of Cd/mol of enzyme was obtained. This rec-$Cd_2$-RPase' exhibited about 89% enzyme activity. However, the reconstitution with $HgCl_2$ yielded enzyme that contained only 1.0 ± 0.3 mol of Hg/mol of enzyme and exhibited 72% RPase activity. Although addition of $1 \times 10^{-5}$ M $CoCl_2$ in buffer D resulted in a renatured enzyme that contained 1.1 ± 0.1 mol of Co/mol of protein, the rec-$Co_1$-RPase' was inactive (4% enzyme activity). As shown in Table IB, the reconstitution in the presence of $CdCl_2$, $HgCl_2$, or $CoCl_2$ generated rec-RPases' containing an insignificant amount of Zn (<0.3 mol/mol of enzyme). On the other hand, when $MnCl_2$, $NiCl_2$, or $CrCl_3$ was used during reconstitution, the reconstituted enzymes did not contain a significant amount of exogenous metal ion (<0.2 mol/mol of enzyme) but high Zn content (0.9~1.2 mol/mol of enzyme) instead. Furthermore, as expected for the rec-$Zn_1$-RPase' (row 1 of Table IB, these one zinc renatured enzymes obtained with $MnCl_2$, $NiCl_2$, or $CrCl_3$ exhibited about 60 ± 10% enzyme activity.

*Role of Sulfhydryl Groups of RPase in Metal Binding.* Results from Table I showed that Cd, Hg, and Zn (all group 12 metals) were readily bound to RPase yielding active enzymes, while other transition metals were poorly incorporated into the renatured enzyme. Since group 12 metals are known to have high affinities for sulfhydryl groups, the role of such groups in metal binding by RPase was studied. The intrinsic Zn ions of the RPase were removed. The free SH groups of the apoenzyme, which may be involved in metal binding, were then either completely or partially blocked with potassium tetrathionate (Yarbrough & Wu, 1974; Wu et al., 1977). An aliquot of the $K_2S_4O_6$-treated enzyme was reacted with DTT to regenerate sulfhydryl groups. Both the blocked and "deblocked" enzymes were then reconstituted in the presence of $CdCl_2$. Metal content of the renatured enzymes was analyzed to determine the effect of sulfhydryl blocking on metal binding.

In the case of "complete" blockage of SH groups, denature apo-RPase was prepared by method I and reacted with $K_2S_4O_6$ by prolonged dialysis against buffer G, which resulted in complete titration of all SH groups in RPase (Yarbrough & Wu, 1974). A portion of the tetrathionate-treated denatured RPase was dialyzed against buffer I to remove the SH-blocking reagent. Both the SH-blocked and free denatured RPases were reconstituted in the presence of $CdCl_2$ by dialysis against buffer H containing $1 \times 10^{-4}$ M $CdCl_2$. Metal content analysis showed that neither reconstituted RPase contained significant amounts of Zn and Cd and both were enzymatically inactive. It appeared that extensive SH blocking had impaired the ability of RPase to bind metal.

The effect of partial blockage of SH groups on metal binding by RPase was also examined. Denatured apo-RPase was prepared according to method II, except that β-ME was omitted from buffer A. To maintain an oxygen-free state in buffer, $N_2$ was continuously purged through the buffer during dialysis. The apoenzyme was incubated with $10^{-2}$ M $K_2S_4O_6$ at room temperature for 30 min. Excess $K_2S_4O_6$ was removed by dialysis against three changes of buffer A without β-ME under $N_2$ atmosphere. A portion of the $K_2S_4O_6$-treated apo-RPase was incubated with $10^{-2}$ M DTT at room temperature for 1 h to free the blocked SH groups. Both the SH-blocked and free RPases were reconstituted by dialysis against buffer D containing $5 \times 10^{-5}$ M $CdCl_2$. Metal analysis

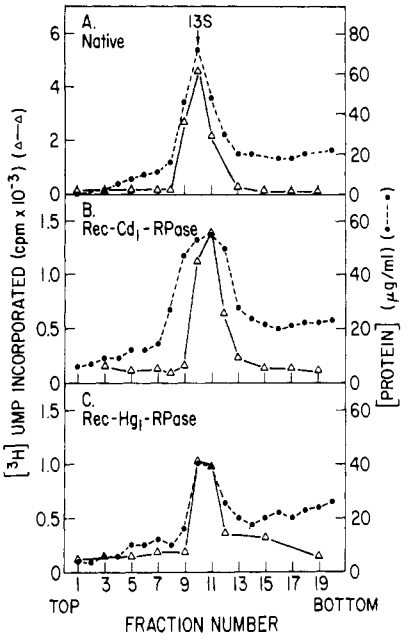


FIGURE 1: Sucrose density gradient sedimentation profiles of native RPase and rec-$Cd_1$- and $Hg_1$-RPases. Both rec-RPases were obtained by method I (solaiman & Wu, 1984). In each gradient, 80–115 μg of RPase was used. Typical percent recovery of enzyme and protein was 20–40% and 60–90%, respectively. (A) Native RPase; (B) rec-$Cd_1$-RPase; (C) rec-$Hg_1$-RPase. (●) Protein concentration; (▲) enzyme activity.

Table II: Abortive Initiation Reactions Catalyzed by Various Reconstituted Enzymes

| enzyme[a] | mol of pppApU synthesized (30 min)$^{-1}$ (mol of enzyme)$^{-1}$ (%)[c] | relative enzyme activity[d] (%)[c] |
|---|---|---|
| native $Zn_2$ RPase[b] | 168 (100) | 100 |
| rec-$Cd_1$-RPase | 101 (60) | 59 |
| rec-$Hg_1$-RPase | 120 (71) | 53 |
| rec-$Cd_2$-RPase' | 110 (66) | 73 |
| rec-$Hg_1$-RPase' | 203 (121) | 137 |

[a] All reconstituted enzymes were further purified by glycerol gradient sedimentation. [b] The specific enzyme activity was 997 units/mg of enzyme. [c] The percent is expressed assuming 100% for native enzyme. [d] Relative total RNA synthesis activities.

of these reconstituted enzymes revealed that they contained no significant amount of Zn but 0.7 ± 0.1 mol of Cd/mol of RPase.

*Biophysical Properties of the Reconstituted Enzymes.* Since the rec-$Cd_1$- or rec-$Hg_1$-RPase exhibited significant enzyme activity (50–70%) and contained one exogenously added metal ion (Table IA), their biophysical and biochemical properties were compared to those of native RPase. As shown in Figure 1, the two rec-$M_1$-RPases sedimented with $S_{20,w}$ = 13 S in a sucrose density gradient—similar to that of native RPase. Similar results were also observed when rec-$Cd_2$-RPase' and rec-$Hg_1$-RPase', obtained by method II, were analyzed by glycerol density gradient sedimentation (data not shown).

The fluorescence spectra of rec-$Cd_1$-RPase and native enzyme were also compared. When rec-$Cd_1$-RPase (0.27 mg/mL) or native RPase (0.24 mg/mL) was excited at 280 nm, the same fluorescence spectra with identical relative intensities (112 vs. 113) at 340 nm were observed. Thus, the gross structures of the two enzymes were very similar.

*Biochemical Properties of the Reconstituted Enzymes.* The ability of various reconstituted enzymes to catalyze abortive initiation reactions was examined. By use of low, nonsaturating

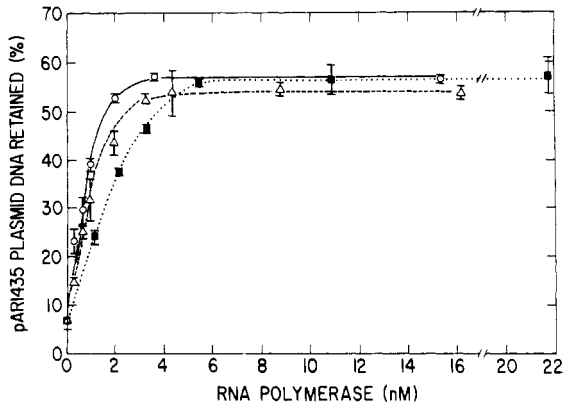


FIGURE 2: Binding of native RPase and rec-$Cd_1$- and $Hg_1$-RPases to $^{32}P$-labeled pAR1435 DNA. Reaction mixture (100 μL) contained 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 10 mM β-ME, 1 mM EDTA, 0.5 mg/mL BSA, 0.8 nM $^{32}P$-labeled pAR1435 DNA [(3.5–4) × $10^8$ cpm/nmol], and various concentrations of enzyme. Incubation was for 10 min at 37 °C. Dilution and filtration were performed as described under Materials and Methods. A negative control (i.e., DNA alone) showed less than 0.5% retention. (○) Native RPase; (△) rec-$Cd_1$-RPase; (■) rec-$Hg_1$-RPase.

Table III: Binding of $^{32}P$-Labeled pAR1435 Plasmid by Reconstituted RPases As Measured by Nitrocellulose Filter Binding Assay

| expt no. | enzyme[a] | binding stoichiometry (mol of enzyme/mol of DNA) | relative DNA binding activity (%) |
|---|---|---|---|
| 1 | native $Zn_2$ RPase | 1.9 | 100 |
| | rec-$Cd_1$-RPase[b] | 2.4 | 79 |
| | rec-$Hg_1$-RPase[b] | 4.3 | 43 |
| 2 | native $Zn_2$ RPase | 1.8 | 100 |
| | rec-$Cd_2$-RPase′[b] | 2.0 | 89 |
| | rec-$Hg_1$-RPase′[b] | 1.6 | 113 |

[a] All reconstituted enzymes were further purified by glycerol gradient sedimentation. [b] The enzyme activities were indicated in Table I.

enzyme concentrations, the rates for the synthesis of the reaction product (pppApU) were measured for each enzyme. The results (Table II) showed that all reconstituted enzymes containing Cd or Hg were able to recognize and initiate RNA synthesis at the A1 early promoter of T7 DNA. The rates of their reactions, ranging from 101 to 203 mol of pppApU synthesized $(30\ min)^{-1}$ (mol of enzyme)$^{-1}$, were comparable to that of native enzyme and corresponded well with the ability of the individual enzyme to catalyze total RNA synthesis (Table I).

The ability of the reconstituted enzymes to bind DNA was next studied by nitrocellulose filter binding assay. Various amounts of enzyme were incubated with $^{32}P$-labeled pAR1435 DNA. The DNA–RPase binary complexes were collected on the filters and quantitated by liquid scintillation counting. Figure 2 shows the DNA binding curves of rec-$Cd_1$-RPase, rec-$Hg_1$-RPase, and native RPase. A control experiment with DNA alone resulted in less than 0.5% retention of the radioactive DNA. The results in Table III showed that while an average of 1.9 mol of native enzyme was needed to retain 1 mol of pAR1435 DNA molecules on the filter, rec-$Cd_1$-RPase and rec-$Hg_1$-RPase, respectively, required an average of 2.4 and 4.3 mol to achieve the same amount of DNA retention. The DNA binding capacities of rec-$Cd_2$-RPase′ and rec-$Hg_1$-RPase′ were also compared with that of native enzyme. Under conditions where an average of 1.8 mol of native RPase caused the retention of 1 mol of DNA, an average of 2 and 1.6 mol of rec-$Cd_2$-RPase′ and rec-$Hg_1$-RPase′ was needed, respectively. These data again showed that the activities of the reconstituted enzymes with respect to DNA template binding closely paralleled their respective abilities to synthesize RNA. The ability of rec-$Cd_2$-RPase′ to bind DNA was comparable to that of native $Zn_2$ RPase.

## Discussion

We recently reported (Solaiman & Wu, 1984) a sequential denaturation–reconstitution method to remove two intrinsic Zn ions from *E. coli* RPase and to reconstitute the apoenzyme to RPase containing one Zn ion with 40–70% enzyme activity recovered. The unique metal ion being incorporated was located in the β subunit and was required for the maintenance of the proper conformation of the active enzyme. In the present study, we have utilized the above method (method I) to study metals other than Zn ion. Among the various metal ions tested, Cd and Hg were able to substitute for Zn in the β subunit of rec-RPase, while Ni and Mn were found to have some or little affinity for the enzyme. Thus, the affinity of β subunit for various metals decreased in the following order: Zn, Cd, Hg > Ni > Mn. Though we had also attempted to reconstitute the apo-RPase with Co and Cr, these metals tended to form aggregates in buffer D, resulting in renatured enzymes containing various amounts of metal. The reason for the formation of aggregates in the reconstitution buffer was unclear—it may be attributed to the susceptibility of these metals to oxidation.

In this study, we also reported a modified procedure of method I for reconstitution of metal-free denatured enzyme. With this new, milder denaturation procedure (method II), we have obtained a renatured RPase containing two Zn ions (rec-$Zn_2$-RPase′) when $ZnCl_2$ was added in buffer D. It was expected that the two Zn ions in rec-$Zn_2$-RPase′ were located in the β and β′ subunits (Wu et al., 1977; Miller et al., 1979). Even in the absence of exogenous $ZnCl_2$ in buffer D, the reconstitution by method II yielded rec-$Zn_1$-RPase′. These results were markedly different from that observed by method I in which apo- and rec-$Zn_1$-RPases were obtained in the absence and presence of $ZnCl_2$ in buffer D, respectively. It appears that the harsh conditions (low-pH treatment and subsequent urea dialysis) in method I destroy the metal binding site in the β′ subunit, and only rec-$Zn_1$-RPase, but not rec-$Zn_2$-RPase, was formed. Furthermore, reconstitution performed by method I without $ZnCl_2$ addition to buffer D yielded apo-RPase, possibly due to the low affinity of the β subunit for Zn. However, the milder denaturation conditions of method II retained both Zn binding sites, and the reconstitution with $5 \times 10^{-5}$ M $ZnCl_2$ yielded rec-$Zn_2$-RPase′. While under Zn-limiting condition (without added $ZnCl_2$ in buffer D), Zn presumably bound only to the high-affinity site in the β′ subunit, and rec-$Zn_1$-RPase′ was obtained. The loss of metal binding capacity of the β′ subunit, but not that of the β subunit by method I, was consistent with the less stable character of the β′ subunit with respect to the other subunits (α, β, and σ) observed in this laboratory. The contention that the β′ subunit may contain a Zn binding site with affinity higher than that found in the β subunit is supported by our earlier studies. When isolated β and β′ subunits were dialyzed separately against buffer containing $1 \times 10^{-5}$ Zn ion for 2 h at room temperature, only 0.6 ± 0.3 mol of Zn was bound to 1 mol of β subunit as compared to 1.4 ± 0.5 mol of Zn/mol of β′ subunit (Wu et al., 1977). Furthermore, with an in vitro metal substitution method developed earlier to prepare reconstituted enzymes, the Zn ion in the β′ subunit remained tightly bound while that in the β subunit could be substituted by exogenous metal ions (Chatterji & Wu, 1982a). The above contention

can be further supported if the subunit location of Zn in the reconstituted enzymes that were obtained under various conditions can be determined. Unfortunately, such comparative studies are not possible at the present time, since isolated subunits tend to lose the intrinsic Zn ions in the presence of urea during subunit separation (Wu et al., 1977).

We found that Cd can substitute both intrinsic Zn ions, while only 1 mol of Hg or Co was incorporated by method II. The reason for the failure of RPase to bind 2 mol of the two latter metals is unclear. One speculation is that after the first Hg or Co bound to RPase a noncooperative binding mechanism was activated by structural changes due to Hg or Co coordination, which prevented the binding of a second metal ion. This is based on the fact that the coordination chemistry of Hg and Co may be different from that of Zn and Cd (Cotton & Wilkinson, 1972). Alternatively, the two metal binding sites in RPase may be in close proximity. The binding of the first Hg with relatively large ionic radius as compared to those of Zn or Cd may sterically hinder the binding of the second Hg ion. Further study on the distance between two metal binding sites and on the nature of Hg or Co binding may shed light on this issue.

$MnCl_2$, $NiCl_2$, and $CrCl_3$ were also employed in reconstitution by method II but failed to be incorporated into the reconstituted RPases that contained one Zn metal ion instead. The results obtained by methods I and II showed that metal binding sites in RPase bound group 12 metals more favorably than other transition metals such as Mn, Cr, and Ni. Since coordination chemistry dictates that group 12 metals bind tightly to sulfhydryl groups, the role of SH groups as ligand(s) in the metal binding sites of RPase was examined. Results from the two SH-blocking experiments suggested that sulfhydryl groups were involved in the metal binding. Complete blocking of these functional groups prevented RPase from binding Cd, but partial blocking allowed the enzyme to bind metal. The lack of enzyme activity in the reconstituted enzymes may be due to the incomplete regeneration of the blocked SH groups by DTT treatment. The possibility that the blocking of a distant SH group(s), which is (are) not liganded to metals, may allosterically affect metal binding cannot be ruled out.

The biophysical and biochemical properties of the reconstituted Cd or Hg RPases obtained by methods I and II were found to be similar to those of native RPase. They assume not only similar quarternary structure as revealed by the same sedimentation coefficients but also similar conformation(s) as shown by fluorescence spectroscopic analysis. Furthermore, the capacities to bind DNA and the rates at which the abortive initiation reactions were catalyzed by the reconstituted enzymes paralleled well their respective activities in total RNA synthesis. These results showed that like Zn (Solaiman & Wu, 1984) Cd and Hg were able to maintain an active, 13*S* conformation for *E. coli* RPase.

The issue of whether the reconstituted $M_1$-RPase is a one-metal enzyme or a mixture of apo-RPase and two-metal enzyme has been discussed in some detail (Solaiman & Wu, 1984). The identity of the rec-$M_1$-RPase as a homogeneous one-metal enzyme was supported by several lines of evidence. For example, the metal content of the reconstituted enzymes purified by DNA–cellulose column chromatography and density gradient sedimentation, and the subunit location of the unique metal being incorporated, both are consistent with the hypothesis.

Our earlier in vivo and in vitro metal substitution studies suggested that the two intrinsic Zn ions were involved in various catalytic and regulatory functions such as DNA and substrate binding, promoter recognition, RNA chain initiation, and coordination of initiation nucleotide. Direct evidence for the catalytic and structural roles of Zn in RPase was subsequently obtained by the removal and readdition of Zn with concomitant inactivation and reactivation of enzyme (Solaiman & Wu, 1984). The same study also showed that Zn in the $\beta$ subunit alone was sufficient to maintain the proper conformation of RPase, enabling the enzyme to catalyze various biochemical reactions. In the present study, we have extended our previous observation with Zn to show that other group 12 metals such as Cd and Hg were capable of replacing Zn in the $\beta$ subunit of RPase to yield an active enzyme. Furthermore, a new milder metal substitution method was also developed to reconstitute RPases containing two Zn or Cd ions but only one Hg. The enzymes reconstituted by method I and II were equally capable of performing various biochemical reactions such as DNA binding, abortive initiation reaction, and RNA synthesis. However, enzymes obtained from method II were quantitatively more reactive than those obtained by method I. Thus, it appeared that although the unique metal in the $\beta$ subunit was sufficient to catalyze those biochemical reactions mentioned above, the intrinsic metal in the $\beta'$ subunit may play a still unknown role to enhance the reactivity of the enzyme. The function of the other metal in the $\beta'$ subunit is currently being investigated. Results from this study not only advance our understanding of the nature of the metal binding sites in RPase but also provide a potential means of investigating the coordination chemistry of the intrinsic metals by using the sensitive and powerful $^{113}Cd$ NMR technique. Furthermore, the possible in vivo role played by the active Cd- and Hg-RPase in the respective expression of Cd- and Hg-resistance operons in *Staphyloccus aureus* (Novick & Roth, 1968) and *E. coli* (Summers & Silver, 1972) can now be studied in vitro.

**Registry No.** RPase, 9014-24-8; Cd, 7440-43-9; Hg, 7439-97-6; Zn, 7440-66-6.

## REFERENCES


Alder, J. F., & Hickman, D. A. (1977) *Anal. Chem. 49*, 336.

Burgess, R. R., & Travers, A. A. (1971) *Methods Enzymol. 21*, 500.

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

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

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

Chatterji, D., Wu, C.-W., & Wu, F. Y.-H. (1984) *J. Biol. Chem. 259*, 284.

Cotton, F. A., & Wilkinson, G. (1972) in *Advanced Inorganic Chemistry*, Interscience, New York.

Hinkle, D. C., & Chamberlin, M. J. (1972) *J. Mol. Biol. 70*, 157.

Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372.

Mildvan, A. S., & Loeb, L. A. (1979) *CRC Crit. Rev. Biochem. 6*, 219.

Miller, J. A., Serio, G. F., Howard, R. A., Bear, J. L., Evans, J. E., & Kimball, A. P. (1979) *Biochim. Biophys. Acta 579*, 291.

Novick, R. P., & Roth, C. (1968) *J. Bacteriol. 95*, 1335.

Oen, H., Wu, C.-W., Haas, R., & Cole, P. E. (1979) *Biochemistry 18*, 4148.

Scrutton, M. C., Wu, C.-W., & Goldthwait, D. A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2497.

Solaiman, D., & Wu, F. Y.-H. (1984) *Biochemistry 23*, 6369.

Speckhard, D. C., Wu, F. Y.-H., & Wu, C.-W. (1977) *Biochemistry 16*, 5228.
Summers, A. O., & Silver, S. (1972) *J. Bacteriol. 112*, 1228.
Wu, C.-W., Wu, F. Y.-H., & Speckhard, D. C. (1977) *Biochemistry 16*, 5449.
Wu, F. Y-H., & Wu, C.-W. (1973) *Biochemistry 12*, 4343.
Wu, F. Y.-H., & Wu, C.-W. (1981) *Adv. Inorg. Biochem. 3*, 143.
Wu, F. Y.-H., & Wu, C.-W. (1983) *Met. Ions Biol. Syst. 15*, 157.
Yarbrough, L. R., & Wu, C.-W. (1974) *J. Biol. Chem. 249*, 4079.

# Elevated Levels of Erythrocyte Hypoxanthine Phosphoribosyltransferase Associated with Allelic Variation of Murine *Hprt*†

Gerald G. Johnson,*,‡ Teresa A. Larsen,‡,§ Patricia Blakely,‡ and Verne M. Chapman∥

*Department of Biology and the Molecular Biology Institute, San Diego State University, San Diego, California 92182, and Department of Molecular Biology, Roswell Park Memorial Institute, Buffalo, New York 14263*

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ABSTRACT: Murine stocks with wild-derived hypoxanthine phosphoribosyltransferase (HPRT) A alleles (*Hprt a*) have erythrocyte HPRT activity levels that are approximately 25-fold (*Mus musculus castaneus*) and 70-fold (*Mus spretus*) higher than those of laboratory strains of mice with the common *Hprt b* allele (*Mus musculus*: C3H/HeHa or C57Bl/6). Since the purified HPRT A and B enzymes have substantially similar maximal specific activities (64 and 46 units/mg of protein, respectively), we infer that these HPRT activity levels closely approximate the relative levels of HPRT protein in these cells. Red blood cells of HPRT A and B mice have similar levels of adenine phosphoribosyltransferase activity (APRT; EC 2.4.2.7) and reticulocyte percentages, which suggests that the elevated levels of HPRT in erythrocytes of HPRT A mice are not secondary consequences of abnormal erythroid cell development. The HPRT activity levels in reticulocytes of HPRT B mice are approximately 35-fold higher than the levels in their erythrocytes and approach the HPRT activity levels in reticulocytes of HPRT A mice. Thus, the marked differences in the levels of HPRT protein in erythrocytes of HPRT A and B mice result from differences in the extent to which the HPRT A and B proteins are retained as reticulocytes mature to erythrocytes. The substantial and preferential loss of HPRT B activity from reticulocytes is paralleled by an equivalent loss of HPRT immunoreactive protein (i.e., CRM) from that cell, and we infer that the HPRT B protein is degraded or extruded as reticulocytes mature to erythrocytes. In studies to be reported elsewhere, we provide evidence that the differences in the levels of HPRT in erythrocytes of HPRT A and B mice are specified by the HPRT structural gene (G. G. Johnson and V. M. Chapman, unpublished results). Thus, the HPRT protein structure is identified as an important factor in determining its susceptibility to turnover in murine erythroid cells.

There is a single hypoxanthine phosphoribosyltransferase (HPRT)[1] structural gene in the mammalian X chromosome (Seegmiller et al., 1967; Miller et al., 1971; Chapman & Shows, 1976). This locus has been widely used as a model system for studies to detect factors that alter gene expression in mammalian somatic cells in culture [Sharp et al., 1975; reviewed by Caskey & Kruh (1979)], since only a single copy of the HPRT gene is expressed in mammalian somatic cells (Rosenbloom et al., 1967; Migeon et al., 1968; Salzmann et al., 1968) and cell culture media are available that can select for either HPRT-plus or HPRT-minus phenotypes in cells (Hakala & Taylor, 1959; Brockman, 1960; Szybalski et al., 1962; Littlefield, 1964).

Few studies, on the other hand, have examined factors that lead to the estimated 200-fold variation in the specific activities of this enzyme in the diverse normal somatic cell types in vivo (Kelley et al., 1969). The reason for the rarity of studies of somatic cell determinants of HPRT expression appears to be that most recognized variants of HPRT represent mutations of the HPRT structural gene which result in the virtual absence of HPRT activity in all cell types (e.g., Lesch–Nyhan syndrome in humans) (Wilson et al., 1983).

We detected electrophoretically distinct forms of HPRT in samplings of feral murine populations which appear to differ from the enzyme deficiency variants described in humans, in that they are common in the populations in which they occur and they are not associated with any obvious deleterious phenotypic effect (Chapman & Shows, 1976; Chapman et al., 1983; G. G. Johnson and V. M. Chapman, unpublished results). In this report, we describe results of studies that compare the levels of HPRT in tissues of mice expressing the wild-derived murine *Hprt a* allele(s) (from *Mus musculus*

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‡ San Diego State University.
§ Present address: Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA 90024.
∥ Roswell Park Memorial Institute.

[1] Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); *Hprt*, the structural gene of HPRT; APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); EDTA, ethylenediaminetetraacetic acid; P-Rib-PP, 5-phosphorylribose 1-pyrophosphate; PEI, poly(ethylenimine); SDS, sodium dodecyl sulfate.

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