Intrinsic Zinc Ion Is Essential for Proper Conformation of Active *Escherichia coli* RNA Polymerase†

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ABSTRACT: The DNA-dependent RNA polymerase (RPase) from *Escherichia coli* contained 2 mol of Zn/mol of holoenzyme ($\alpha_2\beta\beta'\sigma$). An in vitro protocol involving sequential denaturation of RPase in 8 M urea and low pH (2.2), in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA), was developed to completely remove the two intrinsic Zn ions. Subsequent reconstitution of the denatured, Zn-free RPase in the absence and presence of $10^{-5}\sim10^{-4}$ M $ZnCl_2$ yielded respectively the inactive apoenzyme and active (50 ± 10%) RPase containing one Zn ion (rec-Zn_1-RPase). Active rec-Cd_1-RPase was similarly obtained when $CdCl_2$ instead of $ZnCl_2$ was used in the reconstitution. The use of ^{65}Zn as a tracer in the two-step reconstitution procedure showed that the metal was incorporated into renatured enzyme only in the last step of reconstitution. The subunit location of the incorporated metal was identified to be in the β subunit by the use of Affi-Gel Blue column chromatography of rec-Cd_1-RPase. The analysis of apo- and rec-Zn_1-RPases by sucrose density gradient sedimentation showed that the inactive apo-RPase appeared to be consisted of randomly folded protein species with $s_{20,w}$ values ranging from 5 to 18 S, while rec-Zn_1-RPase contained a major, active 13S RPase species and a minor, inactive 7.9S species that could be separated by DNA–cellulose column chromatography. Both 13S and 7.9S RPase contained 1 mol of Zn and the five subunits. The reactivities of 13S rec-Zn_1-RPase in binding DNA and catalyzing abortive initiation reaction were found to be parallel to its activity in total RNA synthesis, whereas apo-RPase was inactive in all these reactions. This study thus employed a direct approach—removal and readdition of intrinsic Zn with concurrent loss and restoration of enzyme activity—to demonstrate that Zn was essential for the proper conformation of active RPase. Our results indicated that the intrinsic Zn played both catalytic and structural roles in RPase.

The DNA-dependent RNA polymerase (RPase)[1] from *Escherichia coli*, an oligomeric enzyme with a subunit composition of $\alpha_2\beta\beta'\sigma$, has been shown to be a Zn metalloenzyme containing 2 mol of Zn/mol of enzyme (Scrutton et al., 1971). One of the two Zn ions is located in the β subunit, which contains the substrate binding site, while the other is in the β' subunit, which possesses the template binding site (Wu et al., 1977; Miller et al., 1979). Although the presence of Zn as an integral part of RPases has been well established in a variety of RNA polymerases isolated from both prokaryotic and eukaryotic sources (Wu & Wu, 1981, 1983), the precise function of the intrinsic metals is still not known and has been a subject of active research in recent years.

The roles of Zn in transcription will be rendered more amenable to investigation if the Zn ions could be substituted with other paramagnetic metals that, unlike Zn ions, possess optical and magnetic properties that are useful in physical studies. We have thus far successfully applied both in vivo (Speckhard et al., 1977; Wu et al., 1977) and in vitro (Chatterji & Wu, 1982a,b; Chatterji et al., 1984) metal substitutions to *E. coli* RPase. In earlier studies with in vivo metal substitution, both Zn ions were replaced with Co(II), and the Co-substituted RPase (Co–Co RPase) was enzymatically as active as Zn–Zn RPase. Comparative studies have revealed that both 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 Co–Co RPase but not the Zn–Zn enzyme has a characteristic absorption spectrum in the visible region that can be perturbed by the addition of substrate and template analogue, suggesting that the intrinsic metal may be involved in the binding of substrate and template to the enzyme (Speckhard et al., 1977). Recently, we have developed an in vitro method that includes sequential denaturation and reconstitution steps to substitute selectively the intrinsic Zn ion in the β subunit with various divalent metal ions (Chatterji & Wu, 1982a). As a result, we have obtained a variety of metal hybrid RPases such as Co–Zn, Mn–Zn, Ni–Zn, and Cu–Zn RPases that exhibit different enzyme activities. Further biochemical and NMR spectroscopic studies indicate that the metal in the β subunit is located at the initiation site of the enzyme and is in direct coordiation (<4 Å) with the base moiety of nucleotide substrate. It thus appears that metal may play a regulatory role in the recognition and orientation of the initiation nucleotide into a stereospecific position for catalysis (Chatterji & Wu, 1982b; Chatterji et al., 1984).

In addition to the metal substitution methods employed above in the studies of the structural and functional role of the intrinsic metals in RPases, a direct way to establish the essential role of the metal in enzyme catalysis is to demonstrate

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[1] Abbreviations: RPase, RNA polymerase; Zn–Zn 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-Zn_1- and rec-Cd_1-RPase, RNA polymerase reconstituted with one Zn and Cd, respectively; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; β-ME, β-mercaptoethanol; NMR, nuclear magnetic resonance; bp, base pair.

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enzyme inactivation by the removal of Zn and subsequent reactivation of enzyme upon Zn replacement. Earlier attempts in removing Zn from *E. coli* RPase by dialysis against EDTA under neutral conditions were not successful (Speckhard et al., 1977) due to extremely tight binding of Zn to the enzyme. Furthermore, prolonged dialysis of the enzyme against 1,10-phenanthroline caused a loss of Zn and a concomitant inactivation of the enzyme not reversible by addition of Zn. Miller et al. (1979) had shown that removal of Zn by prolonged dialysis against 1,10-phenanthroline paralleled the inactivation of enzyme, which could be reactivated by the addition of Zn and Co ions. However, the apoenzyme and the renatured RPase were not isolated and characterized. Moreover, D'-Aurora et al. (1977, 1978) had reported that RPase activity inhibited by 1,10-phenanthroline was due to the formation of an inhibitory phenanthroline–cuprous ion complex in the assay mixture. Thus, a possibility exists that the reactivation observed by Miller et al. (1979) might be due to removal of the inhibitory ligand by the added metals.

Here, we report a direct approach to the preparation of inactive apo-RPase by removal of Zn from RPase under denaturation–low pH conditions and the subsequent reconstitution of the denatured, metal-free enzyme with exogenous Zn to form partially active enzyme containing one Zn ion (rec-Zn_1-RPase). The subunit location of this metal ion was determined to be in the β subunit by the use of rec-Cd_1-RPase. The physical and biochemical properties of the apo-RPase, rec-Zn_1-RPase, and native RPase were compared. The role of intrinsic metals in RPase was also discussed.

Materials and Methods

Materials. Unless otherwise stated, all reagents are of highest quality available commercially and were used without further purification. Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals and ^{32}P- and ^{3}H-labeled nucleoside triphosphates from either ICN Pharmaceutical Co. or New England Nuclear. Calf thymus DNA (type I, highly polymerized) and EDTA were products of Sigma. Ultrapure urea was obtained from Schwarz/Mann, cellulose–acetate electrophoresis strips (Sepraphore III) were from Gelman Sciences, Inc., and the nitrocellulose filter (BA 85) was from Schleicher & Schuell. Affi-Gel Blue resin was purchased from Bio-Rad Laboratories. DNA–cellulose was prepared according to the method of Alberts & Herrick (1971). $CdCl_2$ was obtained from J. T. Baker Chemical Co., $^{65}ZnCl_2$ from New England Nuclear, and β-Fluor from National Diagnostics. Bacto-tryptone and Bacto yeast extract were from Difco Laboratories, and ampicillin was from Sigma.

Enzymes. RPase was purified from *E. coli* MRE 600 cells (Grain Processing) by the method of Burgess & Jendrisak (1975). The enzyme obtained after Bio-Gel A 1.5-m column chromatography was further purified by either phosphocellulose column chromatography in the presence of 50% glycerol (Gonzalez et al., 1977) or DEAE-Sephadex (or DEAE-cellulose) column chromatography under the conditions described by Berg et al. (1971). The enzyme was at least 98% pure as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis and was stored in a storage buffer [0.05 M Tris-HCl (pH 7.8), 0.2 M KCl, 0.1 M $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 60% glycerol] at −20 °C.

The protein concentration of RPase holoenzyme was determined either by the $\epsilon^{1\%}_{280nm} = 6.2$ (Lowe et al., 1979) or by the Coomassie Brilliant Blue dye binding assay (Spector, 1978) with bovine serum albumin (BSA) as standard. The protein concentrations determined by these two methods are in good agreement.

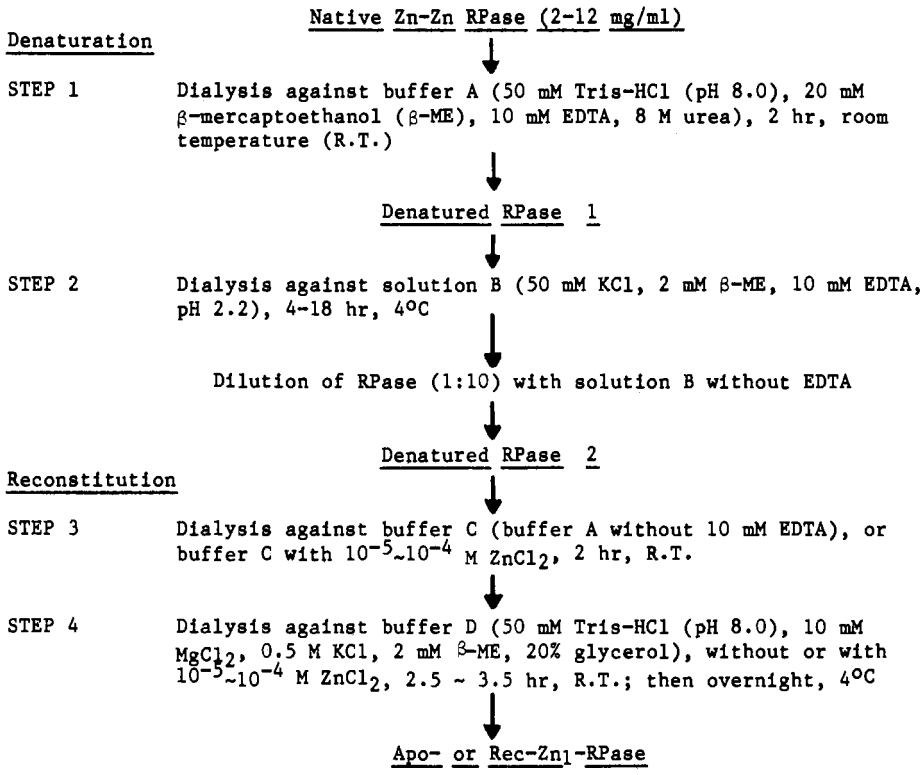
Biochemical Assays. RPase activity was assayed by the incorporation of ^{3}H-labeled ribonucleoside monophosphate into acid-insoluble material with calf thymus DNA as template (Wu & Wu, 1973). One unit of enzyme activity is defined as 1 nmol of [^{3}H]UMP incorporated into the acid-insoluble material in 20 min. Catalase activity was assayed by the decrease in absorbance at 240 nm due to the reduction of H_2O_2 in the reaction mixture as described by Richardson (1966).

Separation of RPase Subunits and Subunit Location of Metal. The core RPase ($\alpha_2\beta\beta'$) and σ subunit were separated by the method of Burgess & Travers (1971) or Lowe et al. (1979). The $\alpha_2\beta$ subunit complex and β' subunit from the core RPase were separated by Affi-Gel Blue column chromatography as described earlier (Wu et al., 1977; Chatterji & Wu, 1982a). The identification of subunits was carried out by electrophoresis on a 7% $NaDodSO_4$–polyacrylamide slab gel (Laemmli, 1970), and protein bands were visualized by the silver staining procedure described by the manufacturer (Bio-Rad Laboratories, Rockville Centre, NY). Alternatively, RPase subunits were separated by electrophoresis on cellulose–acetate sheet (Palm et al., 1975) for 4.5–5 h at 5 mA in a Sepratek chamber (Gelman Sciences, Inc.), with a borate electrophoresis buffer system (0.6 M boric acid, 6 M urea, 0.01 M EDTA, 0.02 M β-ME, adjusted to pH 8.9 with 10 N NaOH) as described by Schafer & Zillig (1973). This latter separation method offered better resolution of β and β' bands than that achieved by $NaDodSO_4$–polyacrylamide slab gel electrophoresis. The metal content of $\alpha_2\beta$ and β' subunits obtained after Affi-Gel Blue column chromatography was determined by atomic absorption spectrometry as described later.

Preparation of Apo-, Rec-Zn_1-, and Rec-Cd_1-RPases. The procedure for the denaturation and reconstitution of RPase is shown in Scheme I. RPase holoenzyme (2–12 mg/mL, 0.2–0.5 mL in storage buffer) was dialyzed against 250 mL of buffer A [50 mM Tris-HCl (pH 8.0), 20 mM β-ME, 10 mM EDTA, and 8 M urea] for 2 h at room temperature. The denatured RPase (denatured RPase 1 in Scheme I) was further dialyzed for 4–18 h at 4 °C against solution B (50 mM KCl, 2 mM β-ME, and 10 mM EDTA, pH 2.2). The enzyme was then diluted 10-fold with solution B without EDTA. The diluted enzyme (denatured RPase 2) was subsequently dialyzed against 250 mL of buffer C (buffer A without EDTA) for 2 h at room temperature followed by dialysis against 500 mL of buffer D [50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.5 M KCl, 2 mM β-ME, and 20% glycerol] for 2.5–3.5 h at room temperature and overnight at 4 °C. When the reconstitution was performed in the presence and absence of exogenous Zn ($10^{-5}\sim10^{-4}$ M $ZnCl_2$ in both buffer C and buffer D), reconstituted Zn enzyme (rec-Zn_1-RPase) and apoenzyme (apo-RPase) were obtained, respectively. rec-$^{65}Zn_1$-RPase was similarly prepared by including $ZnCl_2$ ($10^{-5}\sim10^{-4}$ M) in both buffer C and buffer D and $^{65}ZnCl_2$ (10^3 μCi/mmol) only in buffer D. All reconstituted Zn_1- and apo-RPases were either dialyzed against storage buffer at 4 °C and stored at −20 °C for future use or further analyzed by DNA–cellulose column chromatography as described later. rec-Cd_1-RPase was prepared similarly as described for rec-Zn_1-RPase except $CdCl_2$ instead of $ZnCl_2$ was used.

Sucrose Density Gradient Sedimentation. The sedimentation properties of both apo- and rec-Zn_1-RPases (with or without ^{65}Zn label) were examind by the sucrose density gradient centrifugation according to the method of Martin & Ames (1961), with native RPase as a control and catalase (75 μg, $s_{20,w} = 11.3$) as an internal marker. RPase (45–90 μg in

Scheme I: Preparation of Apo- and Rec-Zn_1-RPases



150–200 μL of storage buffer) was dialyzed against high-salt buffer E [10 mM Tris-HCl (pH 7.9), 0.5 M KCl, 5 mM β-ME, and 0.5 mM EDTA] for 4–5 h at 4 °C. The dialyzed enzyme (180–300 μL) was layered onto a 4.6-mL sucrose density gradient [5–20% (w/v) in buffer E] in a cellulose–nitrate tube. The sedimentation was performed in a Beckman SW-65 rotor at 38000*g* for 17 h at 4 °C. Fractions (250 μL) were collected from the top of the gradient with a Buchler Densi Flow II gradient collector. Protein concentration, enzyme activity, and radioactivity (when rec-$^{65}Zn_1$-RPase was used) of each fraction were measured. The $s_{20,w}$ values for the protein peaks were estimated from the ratios of distances sedimented for sample and standard catalase as described by Gafford et al. (1978). In some experiments, fractions containing the protein peak were pooled and concentrated by vacuum dialysis. The enzyme activity and Zn content of the concentrated sample were compared with those of native RPase.

DNA–Cellulose Column Chromatography. The DNA affinity property of the reconstituted enzymes (rec-Zn_1- or apo-RPase) was analyzed by DNA–cellulose column chromatography. The column was prepared with a DNA to protein ratio of 1.6–2.8 (w/w) and equilibrated with buffer F [10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.1 mM DTT, and 5% glycerol] containing 0.15 M NaCl. The enzyme that had been dialyzed against the buffer F was loaded and incubated on column for 30 min at 4 °C. After the column was washed with the same buffer, protein peak I was obtained. The column was then eluted either with buffer F plus 0.75 M NaCl (4 column volumes) or with a linear gradient of buffer F containing 0.15–0.9 M NaCl (7 column volumes) to yield protein peak II. Fractions (0.2–2.0 mL) were collected, analyzed for metal content, protein concentration, and RPase activity, and analyzed for NaCl concentration by the measurement of conductivity in conductance meter (YSI Model 32, Yellow Springs Instrument Co., Inc.). Peak II RPase was further analyzed by sucrose density gradient sedimentation as described above. For subsequent abortive initiation and DNA binding studies, the peak II RPase was used.

Preparation of Template Plasmid DNA. E. coli strain HMS 174 containing plasmid pAR1435 was kindly provided by Drs. W. Studier and A. H. Rosenberg (Biology Department, Brookhaven National Laboratory, Upton, NY). This plasmid consists of a 102-bp fragment of T7 DNA, including the A1 promoter region, cloned into the *Bam*HI site of pBR322. Bacteria containing the plasmid were grown in LB medium [1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl] containing 40 μg/mL ampicillin. Plasmid DNA was isolated from the cells and purified by CsCl gradient sedimentation in the presence of ethidium bromide as described by Maniatis et al. (1982). The purified DNA was resuspended in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. DNA concentration was estimated spectroscopically with $\epsilon^{1\%}_{260nm} = 200$.

Radioactively labeled pAR1435 used in the nitrocellulose filter binding assay was prepared by the end-labeling method (Hanas et al., 1983). The plasmid DNA was linearized at the unique site with *Eco*RI restriction endonuclease. The protruding ends of the linearized pAR1435 DNA were flushed by the 5′ → 3′ DNA polymerization activity of avian myeloblastosis virus reverse transcriptase with [α-^{32}P]dATP as a substrate. The end-labeled DNA was desalted in a Bio-Gel G-100 column, ethanol precipitated, and resuspended in TE buffer.

Abortive Initiation Assay. The procedure of Oen et al. (1979) was employed, except plasmid DNA (pAR1435) was used as a template. Cocktail A contained 80 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 2 mM DTT, 15 nM plasmid DNA, and various concentrations of RPase (rec-Zn_1- or native RPase) and cocktail B contained 15.6 μM [α-^{32}P]-UTP (360 cpm/pmol), 4.0 mM ATP, 5 mM $MgCl_2$, and 50 mM KCl. After an aliquot (15 μL) of cocktail A was incubated at 37 °C for 10 min, an equal volume of cocktail B was added, and the incubation was allowed to proceed for an additional 30 min at 37 °C. The reaction was terminated by the addition of 10 μL of 0.2 M EDTA, and the entire reaction

mixture was spotted on Whatman 3 MM paper in 5–6-μL aliquots with interim drying. The papers were developed with ascending chromatography in WASP solvent [H_2O/saturated $(NH_4)_2SO_4$ (pH 8.0)/2-propanol, 18/80/2 v/v/v] containing 5 mM EDTA. The chromatograms were dried at room temperature, cut into 1-cm strips, and counted in a liquid scintillation counter. Alternatively, the paper chromatograms were autoradiographed to locate the radioactive spots. These spots were cut from the paper chromatogram and counted. The extent of dinucleotide synthesis was expressed as the ratio between the integrated counts under the dinucleotide peak (R_f 0.46) and the total counts of the entire chromatogram.

Nitrocellulose Filter Binding Assay. The binding assay was performed as described by Hinkle & Chamberlin (1972) except ^{32}P-labeled plasmid DNA pAR1435 was used. The nitrocellulose filters were pretreated according to the procedure of Lin & Riggs (1972) as follows: filters were soaked in 4 N KOH for 20 min, washed 3 times in deionized, distilled water (10 min each), and neutralized in 0.1 M Tris-HCl (pH 7.4) for 30 min. After further being washed twice with water (10 min each), the filters were soaked and kept in washing buffer G [10 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, and 0.2 M NaCl]. RPase (rec-Zn_1-RPase or native RPase) was diluted to 30–40 nM with binding buffer H [10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 10 mM β-ME, and 1 mM EDTA] containing 1 mg/mL BSA. The reaction mixture (0.1 mL) in binding buffer H contained diluted RPase (0–20 nM), 0.5 mg/mL BSA, and 0.8 nM ^{32}P-labeled DNA (1.92×10^5 cpm/nmol). The mixture was incubated for 10 min at 37 °C and then diluted with 1 mL of binding buffer H and immediately filtered through nitrocellulose filter under vacuum (4∼5 in.Hg). The amount of the radioactive DNA retained on filters as a result of RPase binding was measured by liquid scintillation counting.

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 were soaked at least overnight in 30% HNO_3 or 10 mM EDTA and rinsed extensively with deionized, distilled water before use. Prior to measurement, protein (0.1–0.5 mg/mL) was dialyzed at least 16 h at 4 °C against buffer I [10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.1 mM DTT, 0.1 M KCl, and 5% glycerol]. Before Zn analysis, protein was diluted with deionized, distilled water to a protein concentration of 10–50 μg/mL, and 10 μL of solution was introduced into the graphite tube in each determination. Zn content was calculated from the standard curve constructed by using Zn standard solution (Fisher) with deionized, distilled water and buffer I as diluents. Cadmium analysis was similarly performed as Zn analysis except that samples were diluted with 1% (w/v) HNO_3 to yield a protein concentration of 250 μg/mL. The wavelengths used for Zn and Cd determinations were 213.9 and 228.8 nm, respectively. A Searle γ-spectrometer with the window set at 950–1250 keV was used for the measurement of ^{65}Zn concentration.

Results

Preparation of Apo-, Rec-Zn_1-, and Rec-Cd_1-RPases. As shown in Scheme I, *E. coli* RPase holoenzyme was sequentially denatured in 8 M urea buffer A (step 1) and then in pH 2.2 solution B (step 2), both containing 10 mM EDTA. The reconstitution of the denatured enzyme was accomplished by dialysis first against 8 M urea–buffer C without 10 mM EDTA (step 3) and then buffer D without urea (step 4). Table I shows the relative enzyme activities and Zn contents of the reconstituted RPases when Zn(II) (10^{-5}∼10^{-4} M) was either added or omitted in both step 3 and step 4. When $ZnCl_2$ was added in both step 3 and step 4 or in step 4 alone, 50 ± 10% of the RPase activity was recovered as compared to that of the native Zn–Zn RPase, and the Zn content of rec-Zn_1-RPase was found to be 0.95 ± 0.2 mol of Zn/mol of enzyme. However, when $ZnCl_2$ was omitted in steps 3 and 4, the reconstituted enzyme retained only 3% of the original enzyme activity and contained less than 0.2 mol of Zn/mol of enzyme, indicating that apo-RPase was obtained. The same procedure, but with $CdCl_2$ instead of $ZnCl_2$ in steps 3 and 4, was applied successfully to obtain rec-Cd_1-RPase with similar enzyme activity as that of rec-Zn_1-RPase.

The reconstitution of RPase in the presence of Zn was further examined to determine at which stage of reconstitution the exogenous Zn ions were actually incorporated. Table II shows the Zn contents of various enzymes obtained at different steps in Scheme I. The data demonstrated that the intrinsic Zn ions of RPase were effectively removed by treatment with buffer containing 10 mM EDTA under denaturation–low pH conditions and no Zn was associated with denaturated RPase 1 and 2 (steps 1 and 2). Although step 3 in which exogenous Zn ions were introduced in the reconstitution buffer did not play a direct role in Zn incorporation, we found that the omission of this dialysis step failed to yield active, reconstituted

Table I: Zn Content and Enzyme Activity of Apo- and Rec-Zn_1-RPases

expt	addition of $ZnCl_2$ (M) step 3[a]	addition of $ZnCl_2$ (M) step 4[a]	Zn content (mol/mol of enzyme)	enzyme act. (%)[c]
1	*b*	*b*	0.1 ± 0.1	3
	1×10^{-4}	1×10^{-4}	0.8 ± 0.1	50
2	1×10^{-4}	1×10^{-5}	1.2 ± 0.1	35
3	*b*	*b*	0.2 ± 0.1	2
	b	1×10^{-5}	0.8 ± 0.1	66
	b	4×10^{-5}	0.8 ± 0.1	45
	b	1×10^{-4}	0.8 ± 0.2	54
4	5×10^{-5}	1×10^{-4}	1.3 ± 0.1	41

[a] See Scheme I. [b] No addition. However, atomic absorption analysis showed that 2×10^{-6} M Zn was present in the buffer. [c] Enzyme activity was measured in an assay mixture (250 μL) containing 0.08 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 4 mM β-mercaptoethanol, 0.4 mM GTP, ATP, and CTP, 0.4 mM [^3H]UTP (4000 cpm/nmol), 40 μg/mL calf thymus DNA, and 3–10 μg of RPase. Incubation was at 37 °C for 10 min. The activity was expressed as the percent of that of the native Zn–Zn RPase.

Table II: Zn Contents of Samples from Each Step of Denaturation and Reconstitution Procedures (Scheme I) and DNA–Cellulose Column

sample[a]	Zn content (mol/mol of enzyme)	sp act. (units/mg)[d]
Scheme I		
denatured RPase 1	0 ± 0.1[b]	ND[e]
denatured RPase 2	0 ± 0.1[b]	0
step 3 RPase	0.2 ± 0.1[b]	ND[e]
step 4 RPase	1.0 ± 0.1[b]	460
DNA–cellulose column		
peak I RPase[f]	0.9 ± 0.1[b]	
	0.7 ± 0.1[c]	0
peak II RPase[f]	1.2 ± 0.1[b]	690
	0.7 ± 0.1[c]	

[a] Protein concentration of samples were adjusted to 0.1–0.3 mg/mL with buffer. A total of 500 μL of the diluted samples was dialyzed at 4 °C against 1 L of buffer I overnight. [b] Determined by atomic absorption spectrometry. [c] Determined by γ-emission measurement. [d] One unit is defined as the nanomoles of radioactive-labeled nucleoside monophosphate incorporated in 20 min at 37 °C. [e] ND, not determined. [f] Peak I and II obtained from DNA–cellulose column chromatography of rec-Zn_1-RPase, as described in Figure 4.

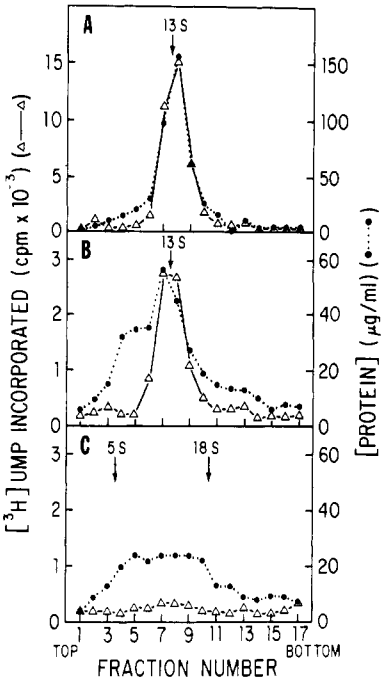


FIGURE 1: Sucrose density gradient sedimentation profiles of native RPase, rec-Zn_1-RPase and apo-RPase. Both rec-Zn_1-RPase and apo-RPase were obtained from Scheme I. A total of 86–93 μg of RPase was used in each gradient. Total recovery of activity and protein was 20–35% and 60–90%, respectively. (A) Native RPase; (B) rec-Zn-RPase; (C) apo-RPase; (●) protein concentration; (△) enzyme activity.

RPase due to precipitation of enzyme. The results also showed that only during reconstitution in step 4 were the exogenous Zn ions bound tightly to the enzyme. A definitive evidence to support this conclusion was provided by subsequent experiments with $^{65}ZnCl_2$ as a tracer in either step 3 or step 4 while both buffer C and buffer D contained nonradioactive $ZnCl_2$ (10^{-5}~10^{-4} M). When $^{65}ZnCl_2$ was added in step 3 as a tracer, no ^{65}Zn radioactivity was detected after step 4 in the final reconstituted RPase. However, this rec-Zn_1-RPase contained 1 mol of Zn/mol of protein by atomic absorption analysis, indicating that 1 mol of Zn was incorporated only in step 4. On the contrary, when $^{65}ZnCl_2$ was present in step 4, ^{65}Zn radioactivity corresponding to 1 mol of Zn/mol of enzyme was detected by γ-emission measurement, which was in good agreement with that obtained by atomic absorption spectrometry.

Sedimentation Properties of Reconstituted Zn_1- and Apo-RPases. The physical properties of the RPases renatured in the presence and absence of exogenous Zn were examined by sucrose density gradient sedimentation. The sedimentation profiles shown in Figure 1 indicated that the rec-Zn_1-RPase appeared to be consisted of two protein species with $s_{20,w}$ values of 7.9 and 13 S (Figure 1B). The major 13S-protein, which had a similar s value as that of the native enzyme (Figure 1A), exhibited ca. 60% enzyme activity as compared to native RPase, while the minor 7.9S-protein, which represented that portion of enzyme incapable of folding into proper conformation of enzyme during reconstitution, was enzymatically inactive. As shown in Figure 1C, the inactive apo-RPase consisted of a spectrum of protein species with $s_{20,w}$ values ranging from 5 to 18 S.

DNA–Cellulose Column Chromatography of Rec-Zn_1-RPase. On the basis of the results from the sedimentation studies, attempts were subsequently made to purify the active RPase fraction from both unlabeled and ^{65}Zn-labeled rec-Zn_1-enzymes by DNA–cellulose affinity column chromatography. Figure 2 shows a typical elution profile when rec-Zn_1-RPase was chromatographed on a DNA–cellulose column. Peak I enzyme, which did not bind to column and was eluted by buffer F containing 0.15 M NaCl, was enzymatically inactive. Peak II enzyme was eluted at 0.35 M NaCl, which was observed for the native RPase chromatographed under the same conditions (data not shown; in a large-scale experiment, it was eluted with a step gradient at 0.75 M NaCl). The specific enzyme activity of peak II RPase was increased by 50% (Table II, enzyme activity rows 4 and 7). Furthermore, peak II RPase appeared mainly as a single protein species in a sucrose density gradient with a $s_{20,w}$ = 13 S (Figure 3B) similar to that for the native RPase in a parallel experiment (Figure 1A), while the inactive peak I protein showed a major peak with $s_{20,w}$ = 7.9 S (Figure 3A). As measured by both atomic absorption spectrometry and ^{65}Zn radioactivity measurement, the Zn contents of peak I and II RPases from the DNA–cellulose column and after sucrose sedimentation (Figure 3) were determined to be 0.8 and 1 mol of Zn/mol of enzyme, respectively (see Table II). These results further confirmed that the rec-Zn_1-RPase obtained in step 4 (Scheme I) was mainly composed of a 13S RPase (with half enzyme activity) and a 7.9S (inactive) component (Figure 1B). The two species appeared to differ in their hydrodynamic properties, resulting probably from different conformations during re-

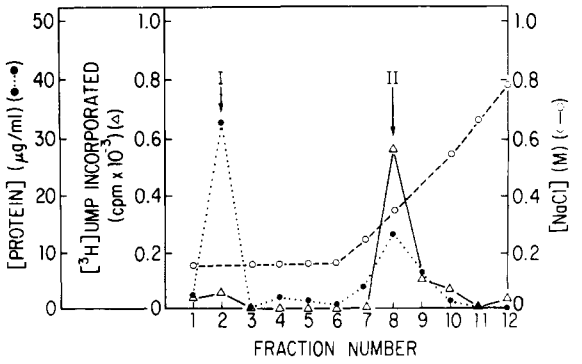


FIGURE 2: Typical elution profile of reconstituted Zn-RPase from DNA–cellulose column chromatography: (●) protein concentration; (△) enzyme activity; (○) NaCl concentration.

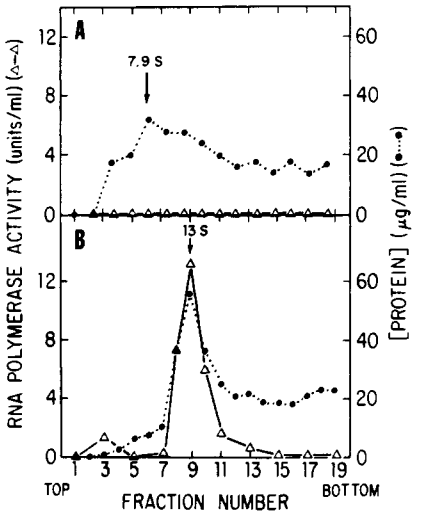


FIGURE 3: Sucrose density gradient sedimentation profiles of peak I and peak II RPases from DNA–cellulose column chromatography of rec-Zn_1-RPase. The enzyme, 66 and 61 μg, from peaks I and II, respectively, was used in the gradient. The profile of the control native RPase (62 μg used) was the same as that in Figure 1A. Total recovery of activity and protein was 20 and 60–90%, respectively. (A) Peak I RPase; (B) peak II RPase; (●) protein concentration; (△) enzyme activity.

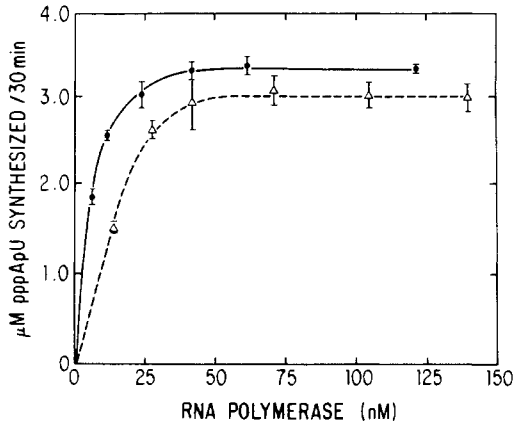


FIGURE 4: Rates of abortive initiation reaction catalyzed by native RPase and rec-Zn_I-RPase. Reaction mixture (30 μL) contained 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 7.5 nM pAR1435 plasmid DNA, 2 mM ATP, 7.8 nM [α-^{32}P]UTP (360 cpm/pmol), and various concentration of RPase. Incubation was for 30 min at 37 °C. (●) Native RPase; (△) rec-Zn_I-RPase.

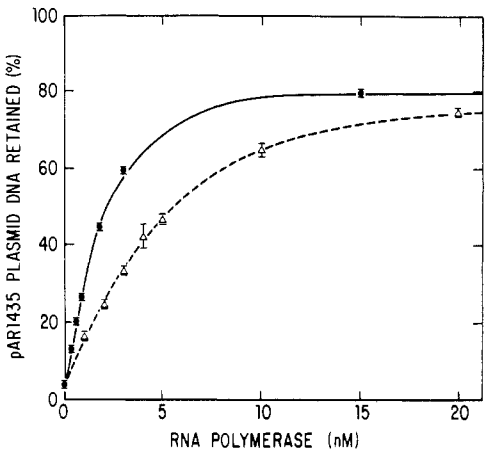


FIGURE 5: Binding of native RPase and rec-Zn_I-RPase to ^{32}P-labeled pAR1435 plasmid DNA. Reaction mixture (100 μL) contained 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.5 mg/ml BSA, 0.8 nM [^{32}P]DNA (1.92×10^5 cpm/nmol), and various concentrations of enzyme. After incubation for 10 min at 37 °C, the reaction mixture was treated as described under Materials and Methods. (●) Native RPase; (△) rec-Zn_I-RPase.

constitution since both contained all RPase subunits (α, β, σ, and β') as shown by NaDodSO$_4$–polyacrylamide gel electrophoretic analysis. Furthermore, results from gel scanning revealed no significant difference in relative abundance of the subunits in these two species as compared to native enzyme. Figure 3 also showed that peak I and II RPases contained protein species with a sedimentation coefficient larger than 13 S. These species are presumably inactive aggregates of the enzymes.

Biochemical Properties of Reconstituted Zn_I- and Apo-RPases. The biochemical properties of rec-Zn_I-RPase (peak II RPase from DNA–cellulose column) and of apo-RPase were further compared with those of the native enzyme. We first studied the ability of the renatured enzymes to catalyze abortive initiation reaction (Johnston & McClure, 1976) by using pAR1435 plasmid DNA as a template. This plasmid contains the A1 promoter with starting sequence of AUCG... from T7 DNA (Siebenlist, 1979). Although pAR1435 also contains promoters P1, P2, and P3 of the pBR322 vector (Brosius et al., 1982), these promoters not only are much weaker in strength in comparison to the A1 promoter of T7 DNA (A. H. Rosenberg, personal communication) but also contain starting sequences that are different from A1 promoter. On the basis of these two reasons, the use of pAR1435 plasmid DNA to study abortive initiation reaction at the A1 promoter site is justified. With this plasmid DNA as template, the relative rates of dinucleoside tetraphosphate (pppApU) synthesis by native RPase and rec-Zn_I-RPase as a function of enzyme concentration were shown in Figure 4. Under non-saturation condition, rec-Zn_I-RPase with 40% enzyme activity in total RNA synthesis catalyzed the abortive initiation reaction at a rate of 109 M pppApU (30 min)$^{-1}$ (mol of protein)$^{-1}$ as compared to 298 M pppApU (30 min)$^{-1}$ (mol of protein)$^{-1}$ for the native RPase. In a separate experiment, the inactive apo-RPase was shown to be incapable of catalyzing the synthesis of pppApU (data not shown). These data indicated that the ability of rec-Zn_I-RPase to catalyze abortive initiation reaction corresponded well with its ability to catalyze total RNA synthesis. At saturation level, the total amounts of pppApU synthesized by both rec-Zn_I-RPase and native enzymes were comparable, indicating that the renatured enzyme recognized and initiated at promoter A1 almost as efficiently as the native RPase.

We next compared the DNA binding ability of the rec-Zn_I-RPase, apo-RPase, and native RPase by using the nitrocellulose filter binding assay. In these experiments, various amounts of enzyme were incubated with ^{32}P-end-labeled pAR1435 DNA. The radioactive DNA–RPase binary complexes were collected on the filters and quantitated by radioactivity measurement. As shown in Figure 5, the stoichiometry for the binding of native RPase to pAR1435 DNA was found to be about 4 mol/mol of DNA, as compared to about 8 for rec-Zn_I-RPase. The DNA binding ability of rec-Zn_I-RPase thus appears to closely parallel to its enzyme activity in total RNA synthesis, which is 40% of that of the native RPase. The maximum DNA binding capacities, shown at saturation level of the curves (Figure 5), are comparable for both renatured and native enzymes, indicating that rec-Zn_I-RPase binds to pAR1435 DNA with similar capacity as that of native RPase. As expected from the enzyme activity, apo-RPase did not bind DNA (data not shown). Results from these biochemical studies indicated that rec-Zn_I-RPase with 1 mol of intrinsic Zn exhibited abortive initiation and DNA binding activities compatible to its activity in total RNA synthesis, whereas the apo-RPase was inactive in all these reactions.

Subunit Location of Metal in Renatured RPase. Since the intrinsic Zn ions in RPase can be released under denaturation conditions (Wu et al., 1977), direct attempt to localize Zn ion in the rec-Zn_I-RPase by the Affi-Gel Blue column chromatography method described previously (Wu et al., 1977) was not possible. Consequently, reconstitution of denatured RPase 2 (see Scheme I) was performed with various transition and heavy metals, in an attempt to obtain a reconstituted RPase in which the metal ion was rather tightly bound. When $CdCl_2$ was used in place of $ZnCl_2$ in such a reconstitution experiment, rec-Cd_I-RPase with enzyme activity comparable to that of rec-Zn_I-RPase was obtained after DNA–cellulose column chromatography. The rec-Cd_I-RPase was then denatured with 7 M urea and chromatographed on an Affi-Gel Blue column as described (Wu et al., 1977; Chatterji & Wu, 1982a). Figure 6 shows the elution profile obtained from this experiment. One Cd ion, as determined by atomic absorption analysis, was found to be associated with the $\alpha_2\beta$ subunit complex (the first protein peak); while the β' subunit (the second protein peak) eluted with high ionic strength buffer was free of metal. Since only β and β' subunits each contained one intrinsic metal (Wu et al., 1977), the results from this experiment indicated that metal in the reconstituted RPase was located in the β subunit.

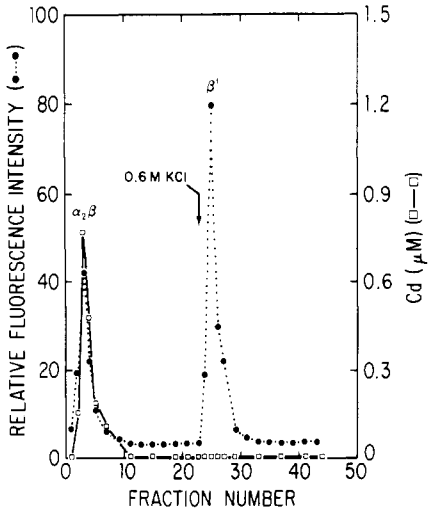


FIGURE 6: Typical elution profile of reconstituted Cd_1-RPase from an Affi-Gel Blue column in the presence of 7 M urea. A total of 830 μL of rec-Cd_1-RPase (0.36 mg/mL) was dialyzed overnight at 4 °C against denaturation buffer J [0.02 M Tris-HCl (pH 8.0), 0.01 M $MgCl_2$, 10% glycerol, and 7 M urea]. The dialyzed enzyme was applied to a 0.5-mL Affi-Gel Blue column preequilibrated with the same buffer. Fractions (0.47 mL) were collected by eluting the column with 10 mL of buffer J ($\alpha_2\beta$ peak) and 10 mL of buffer J plus 0.6 M KCl (β' peak). Fractions were analyzed for Cd content by atomic absorption spectrometry and for protein by measuring the fluorescence at 340 nm with an excitation wavelength of 280 nm.

Discussion

In this study, we have developed a sequential denaturation–reconstitution method to remove two intrinsic Zn ions from *E. coli* RPase and to reconstitute the metal-free denatured enzyme to RPase containing one Zn ion with 40–70% enzyme activity recovered. Results from this study showed that 10 mM EDTA could efficiently extract intrinsic Zn ions once the enzyme was denatured in 8 M urea followed by dialysis at low pH (pH 2.2). Early attempts to remove two Zn ions by 10~100 mM EDTA under neutral and nondenaturating conditions were unsuccessful (Speckhard et al., 1977; Miller et al., 1979), and a prolonged dialysis of the enzyme against 1,10-phenanthroline caused a loss of Zn and a concomitant inactivation of the enzyme not reversible by addition of Zn (Speckhard et al., 1977). Though Miller et al. (1979) reported that apo-RPase prepared by prolonged dialysis against 10 mM 1,10-phenanthroline could be reactivated by dialysis against buffer containing Zn or Co, the apoenzyme had not been isolated, and the reconstitution process and the property of the renatured enzyme were not characterized. Furthermore, the complex formed between 1,10-phenanthroline and cuprous ions, which is a common contaminant in the enzyme assay solution, was known to inhibit DNA and RNA polymerases (D'Aurora et al., 1977, 1978; Sigman et al., 1979). Thus, it was not clear whether the reactivation of apoenzyme in the earlier study (Miller et al., 1979) was due to reassociation of Zn ions with apoenzyme or to the removal of the inhibitory ligand by the addition of exogenous metal (Ferrin et al., 1983). With the successful employment of EDTA as a metal scavenger in this study, the complications caused by the use of 1,10-phenanthroline were circumvented.

The effectiveness of EDTA in complete removal of intrinsic Zn ions of *E. coli* RPase was demonstrated with the aid of two factors: the denaturation of the multisubunit enzyme by urea and the competition for the cation binding sites by protons (low pH conditions) (Petering, 1972; Rossotti & Rossotti, 1961). Under these conditions, the enzyme was rendered metal free within 6 h. In contrast to this short dialysis period, previous methods (Scrutton et al., 1971; Miller et al., 1979) required days of dialysis, which resulted in enzyme inactivation. The present method had avoided the use of not only the inhibitory ligand 1,10-phenanthroline but also the undesirable prolonged dialysis.

Once the intrinsic Zn ions had been removed, the reconstitution of RPase activity had an absolute requirement for the presence of metals. In the absence of exogenous Zn ions during reconstitution, less than 2–3% of the enzyme activity was recovered. On the contrary, the presence of 10^{-5}–10^{-4} M $ZnCl_2$ resulted in up to 70% recovery of enzyme activity. The possibility that such reactivation is due to removal of inhibitory ligand as suggested from the reconstitution of *E coli* DNA polymerase I (Ferrin et al., 1983) is unlikely. First, not only the formation of cuprous–EDTA complex is unfavorable (Sillen & Martell, 1964) but also the inhibitory effect of such complex had never been documented for DNA-dependent RPase reactions. Second, our results showed that the exogenously added Zn ions were indeed tightly incorporated into the renatured enzyme (see Table II and results of the $^{65}ZnCl_2$ experiments). These observations directly demonstrated for the first time that Zn played an essential role for RNA polymerase activity.

An earlier report (Yarbrough & Hurwitz, 1974) seemed to indicate that the addition of $ZnSO_4$ to the reconstitution buffer had no effect on the recovery of enzyme activity during reconstitution of individual subunit into holo or core RPase. Precise reasons for their observations were not clear. One possibility might be that the intrinsic Zn ions remained loosely bound to the denatured enzymes under the experimental conditions, and they were immediately reincorporated into the protein during reconstitution. Consequently, the added, exogenous Zn did not play any role in the reactivation process. Results from our earlier in vitro metal substitution procedure (Chatterji & Wu, 1982a) seemed to support this notion. In these studies, the β' subunit of the hybrid enzymes (such as Co–Zn or Mn–Zn RPase) still contained Zn ion that may be derived from the intrinsic Zn loosely associated with the protein throughout the course of denaturation and reconstitution. The present study, however, clearly showed that we obtained denatured RPase containing no Zn and such metal-free enzyme required Zn for its reconstitution to active species.

In addition to its catalytic role in *E. coli* RPase, Zn also exhibited a structural role from our sucrose density gradient sedimentation studies. The sedimentation profile of rec-Zn_1- and apo-RPases from step 4 in Scheme I differed significantly. The subunits of apoenzyme appeared to assemble in a random manner yielding an even distribution of oligomeric protein species with $s_{20,w}$ values ranging from 5 to 18 S (Figure 1C). Thus, the absence of Zn attributed to this random assembly of subunits and inactiveness of apo-RPase. In contrast, the presence of exogenous Zn ions had shifted the equilibrium from the random fashion of subunit association to the orderly, active conformation of the 13S enzyme, with the inactive 7.9S species appearing as a minor component (Figure 1B). When the rec-Zn_1-RPase was further purified through DNA–cellulose column chromatography, 13 and 7.9S species were separated. The 13S RPase that remained as a single species after further sedimentation analysis (Figure 3B) contained 1 mol of Zn/mol of enzyme and exhibited 50–60% activity, whereas the 7.9S species though possessing 1 mol of Zn did not have enzyme activity due to incorrect conformation of protein. Though the basis for the formation of two enzyme species (DNA–cellulose column peak I and II RPases) each with all the subunits and

one Zn, but different properties, is not readily apparent, these results provided the first strong evidence for the involvement of Zn ions in determining the quaternary structure of an active RPase.

We have routinely obtained rec-Zn_1-RPase or rec-Cd_1-RPase with 40–70% recovery of enzyme activity, even though a higher percentage had been reported for "conventional" (not emphasizing metal status) reconstitution from individual subunit (Zillig et al., 1970; Ishihama & Ito, 1972). One might speculate that the two metal binding sites in RPase might be similar, and as a result, the renatured enzyme with only one metal would possess partial enzyme activity. However, results from our abortive initiation and DNA binding studies showed that at saturation condition the rec-Zn_1-RPase exhibited maximal rates comparable to those of the native RPase. It appeared that the renatured enzyme contained an active population that was similar to the native RPase with respect to the activity in abortive initiation, DNA binding, and total RNA synthesis. The lower percent recovery of enzyme activity in this study might be due to the harsh conditions [such as 8 M urea followed by low pH (2.2) treatment] experienced by the protein during the denaturation–reconstitution process. This rationale seemed to be supported by results from our earlier in vitro metal substitution studies (Chatterji & Wu, 1982a) with milder denaturation conditions (8 M urea without low pH treatment). In this cited study, the reconstituted Zn–Zn RPase recovered only 66% of the enzyme activity. Preliminary work to obtain metal-free, denatured RPase by use of 8 M urea–10 mM EDTA dialysis without subsequent treatment with low pH (2.2) was successful. This milder condition for the removal of intrinsic metals has enabled us to reconstitute an active, native RPase containing two metals (unpublished results).

The identity of the rec-Zn_1-RPase as a one-zinc enzyme, not a mixture of apo-RPase and Zn–Zn RPase, is supported by several lines of evidence. First, the Zn content of the renatured enzyme remained 1 mol/mol of protein throughout the two purification steps involving DNA–cellulose column chromatography and sucrose density gradient sedimentation. When apo-RPase was used as control in a similar experiment, only a small amount of the protein was found to be either bound to DNA–cellulose column or sedimented with a coefficient of 13 S. Consequently, if rec-Zn_1-RPase were a mixture of apo-RPase and Zn–Zn RPase, the Zn–Zn enzyme would have been obtained after the two purification steps. The fact that only the one-zinc enzyme was obtained following DNA–cellulose chromatography and sucrose density gradient sedimentation indicated that the active rec-Zn_1-RPase was a homogeneous one-metal species. Additional evidence to support this contention was provided by the results from the Affi-Gel Blue column chromatography of rec-Cd_1-RPase. As shown in Figure 6, one Cd was found to be associated only with the β, but not β', subunit. The results strongly indicated that a Cd–Cd enzyme might not exist in the rec-Cd_1-RPase, and the reconstituted enzyme was a one-cadmium species.

Since the first discovery of two tightly bound Zn ions in *E. coli* RPase, their importance to the function of the enzyme had been the subject of many studies. On the basis of the results obtained from our earlier in vivo and in vitro studies (Speckhard et al., 1977; Chatterji & Wu, 1982a,b), the two intrinsic metals had been implicated in various catalytic and regulatory functions such as DNA and substrate binding, promoter recognition and RNA chain initiation, and coordination of initiation nucleotide. These results indirectly suggested that the two intrinsic metals in *E. coli* RPase were important for the enzyme activity. In this study, the role of intrinsic metals in *E. coli* RPase was directly demonstrated by the removal and readdition of Zn with concomitant inactivation and reactivation of enzyme. We showed for the first time that Zn-free, denatured RPase could be isolated and reconstituted into active enzyme only in the presence of metal, providing direct proof for the catalytic role of the intrinsic metal in RPase. In addition, our results suggested that Zn ion in the β subunit also played a structural role in maintaining the proper conformation of active RPase. The reconstitution method developed in this study can be used to prepare a series of metal-substituted RPases for further study on the role of intrinsic metals in RPases. The function of the other metal in the β' subunit is currently being investigated.

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Isolation and Identification of a Tyrosyl Peptide Labeled by 5′-[*p*-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine at a GTP Site of Glutamate Dehydrogenase†

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ABSTRACT: The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBεA) was shown previously to react at a GTP inhibitory site on bovine liver glutamate dehydrogenase. The incorporation was limited to 1.28 mol of reagent/mol of subunit and was attributed to 0.95 mol of modified tyrosine/mol of subunit and 0.33 mol of modified lysine/mol of subunit, quantitatively accounting for the total incorporation prior to acid hydrolysis [Jacobson, M. A., & Colman, R. F. (1983) *Biochemistry 22*, 4247–4257]. The specific tyrosyl peptide modified by 5′-FSBεA has been isolated from a tryptic and chymotryptic digest of modified enzyme by gel filtration and reverse-phase high-performance liquid chromatography and characterized by amino acid and amino-terminal analysis. A unique residue, tyrosine-262, was identified as an essential amino acid within the GTP binding site. The stacked conformation of the fluorescent analogue when enzyme bound suggests that tyrosine-262 may be located in the region of the GTP site which binds the purine ring.

The activity of the allosteric enzyme bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is modulated by GTP, which inhibits, ADP, which activates, and NADH, which inhibits at high concentrations by binding at a site distinct from the catalytic site (Goldin & Frieden, 1972). The enzyme is composed of six identical subunits with several nucleotide sites per subunit, including one for ADP, two for GTP, and two for NADH (one catalytic and one regulatory) (Goldin & Frieden, 1972; Pantaloni & Dessen, 1969; Pal & Colman, 1979). In the attempt to identify essential amino acid residues in the regulatory sites of glutamate dehydrogenase, the reactions of several purine nucleoside affinity labels with the enzyme have been studied (Colman, 1983). The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBεA)[1] has been shown to react specifically at one of the GTP sites on glutamate dehydrogenase (Jacobson & Colman, 1982) and has been utilized as a fluorescent probe in the estimation of distances by fluorescence energy transfer between this site and the ADP activator site (Jacobson & Colman, 1983). As compared to native glutamate dehydrogenase, modified enzyme retains full catalytic activity and normal ability to be inhibited by high concentrations of NADH but exhibits a decreased affinity for and diminished maximum inhibition by saturating concentrations of GTP and a decreased maximum extent of activation with no change in affinity for ADP. Enzyme with 1.28 mol of 5′-(*p*-sulfonylbenzoyl)-1,N_6-ethenoadenosine incorporated/mol of subunit and exhibiting maximum change in sensitivity to GTP inhibition has been shown to contain 0.95 mol of *O*-[(4-carboxyphenyl)sulfonyl]tyrosine (CBS-Tyr) and 0.33 mol of N^ϵ-[(4-carboxyphenyl)sulfonyl]lysine (CBS-Lys), quantitatively accounting for the total incorporation prior to acid hydrolysis (Jacobson & Colman, 1983). As a function of time of incubation with 5′-FSBεA, the amount of CBS-Tyr formed was directly proportional to the change in GTP inhibition. In contrast, an initial formation of CBS-Lys was observed, followed by relatively little additional CBS-Lys, although the percent change in GTP inhibition continued to increase. Thus, it was concluded that the tyrosine is an essential residue in the GTP binding site of glutamate dehydrogenase, while the

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[1] Abbreviations: 5′-FSBεA, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; 5′-SBεA, 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine; CBS-Tyr, *O*-[(4-carboxyphenyl)sulfonyl]tyrosine; CBS-Lys, N^ϵ-[(4-carboxyphenyl)sulfonyl]lysine; HPLC, high-performance liquid chromatography; TPCK, tosylphenylalanine chloromethyl ketone.

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