Selective Substitution in Vitro of an Intrinsic Zinc of *Escherichia coli* RNA Polymerase with Various Divalent Metals†

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ABSTRACT: A simple in vitro substitution method involving a sequential denaturation–reconstitution process was developed to substitute selectively one of the two intrinsic Zn ions in *Escherichia coli* RNA polymerase with Co, Mn, Ni, or Cu ion. The resultant metal hybrid Co–Zn, Mn–Zn, Ni–Zn, and Cu–Zn RNA polymerases possess 100, 100, 60, and 17% of the enzymatic activity of the reconstituted Zn–Zn enzyme, respectively. The substituted metal was found to be located in the β subunit of the polymerase which contains the substrate binding site. The biochemical and physical properties of these metal-substituted polymerases were compared with those of the native Zn enzyme. Co–Zn and Ni–Zn core polymerases exhibit characteristic absorption spectra in the near-UV and visible region, while Mn–Zn and Cu–Zn enzymes do not. The Co–Zn enzyme shows two major peaks at 400 nm (ϵ = 3000) and 475 nm (ϵ = 2700), while the Ni–Zn enzyme exhibits a major peak at 462 nm (ϵ = 8000). The difference absorption spectrum of Ni–Zn core polymerase could be perturbed by the addition of substrate ATP but not by UTP in the absence of template and Mg(II) ion. These observations suggest that the substituted metal was located at the initiation site of the enzyme. The various metal hybrid enzymes do not differ appreciably in their abilities to incorporate noncomplementary nucleotide or deoxyribonucleotide into RNA product. It was found, however, that the difference in enzymatic activities of these metal hybrid enzymes resides at least partly in the initiation step of RNA synthesis.

It has been known that a variety of nucleotidyl transferases including DNA and RNA polymerases from both prokaryotic and eukaryotic sources are Zn metalloenzymes (Mildvan & Loeb, 1979; Wu & Wu, 1981). The *Escherichia coli* DNA-dependent RNA polymerase (RPase)[1] contains 2 mol of Zn/mol of holoenzyme which has a subunit composition of $\alpha_2\beta\beta'\sigma$ (Scrutton et al., 1971). One of the two Zn ions is located in the β subunit, whereas the other is in the β' subunit (Wu et al., 1977; Miller et al., 1979). While the presence of Zn as an integral part of RPase has been well established, the precise function of the intrinsic metal remains to be elucidated. One method to probe the role of intrinsic Zn in RPase is by substituting it with paramagnetic metals which possess optical and magnetic properties that are useful for physicochemical studies. Such a method has been applied successfully to a number of Zn metalloenzymes (Lindskog, 1970).

Previously, we replaced the two intrinsic Zn ions in *E. coli* RPase in vivo with Co(II) by growing the bacteria in a Zn-depleted Co-enriched medium (Speckhard et al., 1977). The enzyme purified from these cells contains 2 mol of Co/mol of enzyme with concomitant reduction in the Zn content. The Co-substituted RPase (Co–Co RPase) is enzymatically as active as Zn RPase (Zn–Zn RPase) on a variety of templates. Comparative studies have revealed that the Co-substituted and native enzymes are very similar both biochemically and physically except that the former has a characteristic absorption spectrum in the visible region. This visible spectrum is perturbed by addition of nucleoside triphosphates or a template analogue, $d(pT)_{10}$, suggesting that the intrinsic metal may be involved in the binding of substrate or template to the enzyme. It is not clear, however, whether one or both of the intrinsic metal ions are involved in the binding process. Moreover, a question may be raised whether the spectral changes observed are due to a direct participation of the intrinsic metal in the binding or to an indirect effect such as conformational changes of the enzyme induced by substrate or template binding at sites away from the intrinsic metal. This uncertainty can be resolved if the distance between the metal and the substrate (or template) binding sites on the enzyme is known. Because Co(II) is a paramagnetic metal, such a distance can, in principle, be determined by NMR or EPR spectrometry (Mildvan et al., 1980; James, 1975). However, for the biosynthetically prepared Co–Co RPase, the distance measurements will be complicated by the presence of two Co ions on the enzyme. Selective replacement of a single intrinsic metal is necessary for assignment of a specific role and for elucidation of the spatial relationship between the metal binding site and other active sites of the enzyme.

The simplest way to achieve the specific metal substitution in metalloenzymes is to replace or to exchange the intrinsic metal with another metal in vitro. This was difficult for *E. coli* RPase because of the extremely tight binding of Zn to the enzyme. These Zn ions could neither be removed from RPase by prolonged dialysis against metal chelators nor be exchanged with other divalent metal ions present in dialysis solutions (Speckhard et al., 1977). In this paper, we describe a simple denaturation–reconstitution method to substitute selectively one of the two Zn ions in *E. coli* RPase, which is located in the β subunit. This intrinsic Zn was replaced by Co(II), Mn(II), Ni(II), and Cu(II) to yield various metal hybrid enzymes, Co–Zn, Mn–Zn, Ni–Zn, and Cu–Zn RPase,

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Affi-Gel Blue, Cibacron Blue dye covalently cross-linked to agarose; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; 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; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

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respectively. Both physical and biochemical properties of these metal hybrid enzymes were compared with those of the native enzyme (Zn–Zn RPase). NMR studies on the spatial relationship between the metal and substrate binding sites using Co–Zn RPase will be reported in a subsequent paper.

Experimental Procedures

Materials. Metal standards and salts were obtained from Fisher Scientific Co. Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals. ^{3}H- and α-^{32}P-labeled nucleoside triphosphates and ^{57}Co ($CoCl_2$, 10 mCi/mL) were obtained from New England Nuclear. Ultrapure urea, Tris, and sodium dodecyl sulfate were obtained from Schwarz/Mann. Phosphocellulose (P 11) was supplied by Whatman. Affi-Gel Blue (100–200 mesh) and Chelex-100 were obtained from Bio-Rad. Calf thymus DNA was the product of Worthington. T7 DNA and T7 ΔD111 DNA were prepared as described by Hillel & Wu (1978) except that the phage was grown in *E. coli* at 37 °C in the medium of Fraser & Jerrel (1953) in a 10-L fermenter (New Brunswick, NJ). The ΔD111 deletion of T7 phage strain was kindly provided by Dr. F. W. Studier of Brookhaven National Laboratory. All other biochemicals were of highest purity obtained commercially and used without further purification.

Enzymes. RPase was purified from *E. coli* MRE 600 by the method of Burgess & Jendrisak (1975). The enzyme was at least 98% pure as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and was stored in a storage buffer [0.05 M Tris-HCl (pH 7.8), 60% glycerol, 0.2 M KCl, 0.1 M $MgCl_2$, 10^{-4} M EDTA, and 10^{-3} M dithiothreitol] at −20 °C. The holoenzyme used in abortive intiation was obtained by incubating the reconstituted core enzyme with an equimolar amount of σ subunit at 37 °C for 5 min. The RPase subunits were isolated by the procedure of Yarbrough & Hurwitz (1974). The core RPase ($\alpha_2\beta\beta'$) and the σ subunit were separated by the method of Burgess & Travers (1971) or Lowe et al. (1979). The protein concentrations of holo and core RPase and of the σ subunit were determined by the extinction coefficients ($\epsilon^{1\%}_{280nm}$) of 6.2, 5.5, and 8.4, respectively (Lowe et al., 1979). The separation of the subunit complex $\alpha_2\beta$ and the β' subunit from the core RPase by Affi-Gel Blue was performed as described earlier (Wu et al., 1977).

Biochemical Assays. RPase activity was assayed by the incorporation of ^{3}H-labeled ribonucleoside monophosphate into acid-insoluble material by using calf thymus DNA as template (Wu & Wu, 1973). The σ subunit was assayed by its stimulatory effect on the DNA-dependent RNA synthesis by core RPase by using T7 DNA as template following the procedure of Yarbrough & Wu (1974). The fidelity of the metal-substituted RPases in gene transcription was performed by the method of Springgate & Loeb (1975). The assay procedure of Oen et al. (1979) was used to examine the abortive initiation reactions of metal-substituted RPases with T7 ΔD111 DNA as template.

In Vitro Substitution of Zn in Core RPase with Other Metals. Core RPase (5–10 mg/mL) was denatured by dialysis for 2–3 h at 23 °C against denaturation buffer [0.05 M Tris-HCl (pH 8), 10^{-2} M dithiothreitol, 0.1 mM EDTA, 5% glycerol, and 7 M urea] containing 10^{-4}–10^{-5} M Co(II) [or ^{57}Co(II), $t_{1/2}$ = 270 days), Mn(II), Ni(II), or Cu(II). The denatured enzyme was then reconstituted by dialysis for 3 h at 23 °C and then overnight at 4 °C against reconstitution buffer [0.05 M Tris-HCl (pH 8), 0.2 M KCl, 0.01 M $MgCl_2$, 1 mM EDTA, 0.02 M β-mercaptoethanol, and 20% glycerol] containing 10^{-4}–10^{-5} M divalent metal ion. The excess or loosely bound metal ions in reconstituted core RPase were removed by dialysis for 24 h at 4 °C against buffer A [0.01 M Tris-HCl (pH 8), 10 mM EDTA, 0.1 M KCl, 0.1 mM dithiothreitol, and 5% glycerol]. The protein thus obtained was stored in storage buffer after precipitation with ammonium sulfate. The same experiment as described above was performed as a control except that the exogenous divalent metal was Zn instead of other metals. These experiments were carried out in disposable plastic flasks to avoid contamination of metals from glassware. For the control experiment for the ^{57}Co substitution, the core RPase without denaturation was dialyzed against reconstitution buffer containing the same amount of ^{57}Co.

Oxidation of Co(II) in RPase to Co(III) with H_2O_2. The ^{57}Co(II)-substituted core RPase (0.25 mg, 8×10^4 cpm) mixed with 1 mg of Co(II) core enzyme was treated with a 20-fold molar excess of H_2O_2 at room temperature for 1 h as described by Wu et al. (1977).

Metal Determination. Metal content was determined by using a Perkin-Elmer Model 4000 atomic absorption spectrophotometer equipped with an HGA 400 graphite furnace. Before use, all labware was acid washed and rinsed with deionized, distilled water. Measurements were standardized by the Fisher metal standards. Prior to measurement, protein was dialyzed at least 16 h at 4 °C against buffer A to remove excess or loosely bound metal and was adjusted to 0.1–0.2 mg/mL. For Co, Ni, or Cu RPase, the enzyme was diluted with 0.01 N nitric acid which was used as a control matrix, and water was used as a control for the Zn or Mn RPase. The wavelengths used in the absorption spectrophotometer for determination of Zn, Mn, Co, Ni, and Cu were 213.9, 279.5, 240.7, 232.0, and 324.7 nm, respectively.

Absorption Spectroscopy. The absorption spectrum was measured in a 0.5-mL masked quartz cuvette by using a Cary 118 spectrophotometer equipped with a scatter-transmission accessory. The cell compartment was thermostated at 23 °C.

Results

In Vitro Substitution of Intrinsic Zn with Various Divalent Metals. As mentioned before, it was difficult to substitute the intrinsic Zn in *E. coli* RPase in vitro with other metals. The Zn could not be removed by prolonged dialysis against 10 mM EDTA in 0.05 M Tris-HCl buffer (pH 8) containing 0.1 M KCl, 1 mM β-mercaptoethanol, and 5% glycerol, even over a period of 2 weeks. Prolonged dialysis of the enzyme with 1,10-phenanthroline in the same buffer resulted in a loss of Zn and an irreversible loss of enzyme activity. Furthermore, the tightly bound Zn could not be exchanged with Co(II), Cd(II), Cu(II), or Mn(II) ions present in the dialysis solution. Therefore, we have attempted to achieve the in vitro substitution by a sequential denaturation–reconstitution method, the details of which are described under Experimental Procedures. Purified core RPase ($\alpha_2\beta\beta'$) was partially denatured with 7 M urea to remove the tightly bound Zn. The denatured core enzyme was then reconstituted by dialysis against reconstitution buffer containing 10^{-5} M Co(II), Mn(II), Ni(II), or Cu(II) ions. After removal of excess or loosely bound metal ions, the reconstituted core enzyme was converted into holoenzyme by addition of isolated σ subunit. Core enzyme rather than holoenzyme was used as starting material in the in vitro substitution because the two Zn ions are in the core enzyme and the isolated σ subunit is essentially free of Zn (Wu et al., 1977). Besides, the method for reconstitution of denatured core RPase into active enzyme has been developed (Yarbrough & Hurwitz, 1974).

Table I shows the enzymatic activities of the native and reconstituted core polymerases in the presence and absence

Table I: Specific Activities of Native and Reconstituted Core RNA Polymerases in the Presence and Absence of σ Subunit Using T7 DNA as Template[a]

reconstitution	enzyme activity (units/mg) $-\sigma$	$+\sigma$	$+\sigma/-\sigma$
no (native enzyme)	347 (100)[b]	1736 (100)	5.0
reconstituted in Zn(II)	181 (52)	1145 (66)	6.3
reconstituted in Co(II)	208 (60)	1215 (70)	5.8
reconstituted in Mn(II)	205 (59)	1180 (68)	5.8
reconstituted in Ni(II)	109 (30)	710 (41)	6.5
reconstituted in Cu(II)	60 (17)	185 (11)	3.0

[a] One unit is defined as the nanomoles of radioactive-labeled nucleoside monophosphate incorporated in 20 min at 37 °C. [b] Values in parentheses are given in percent.

of the σ subunit with T7 DNA as template. Under our experimental conditions, core RPase reconstitution in the presence of Zn(II) ion regained 50–70% of the initial enzymatic activity. The specific activities of core enzyme reconstituted in the presence of other divalent metals were in the order Zn = Co = Mn > Ni > Cu. Addition of the σ subunit to reconstituted core enzymes produced a 5–6-fold increase in the specific activity, similar to the extent of σ subunit stimulation observed for native core enzyme by using the same template. The reconstituted Co, Mn, Ni, and Cu holoenzymes (by adding σ subunit to the corresponding core enzymes) possess 100, 100, 60, and 17%, respectively, of the specific activity of the reconstituted Zn holoenzyme.

The metal contents of the native and reconstituted core RPases determined by atomic absorption spectrometry are presented in Table II. Both native and Zn-reconstituted core enzymes contain 2 μmol of Zn with negligible amounts of other divalent metals measured. For the enzyme preparations reconstituted in Co, Mn, Ni, or Cu, an analysis of their metal contents indicates that in all cases only one of the two Zn ions has been replaced by the corresponding divalent metal. This stoichiometry was confirmed by the radioactivity measurement of the core polymerase reconstituted in the presence of ^{57}Co(II). Thus, by the denaturation–reconstitution method, we have successfully substituted one of the two intrinsic Zn ions in RPase to yield the Co–Zn, Mn–Zn, Ni–Zn, and Cu–Zn metal hybrid enzymes.

Subunit Location of the Substituted Metal. The question as to which of the two Zn ions was substituted in the metal hybrid enzyme was answered by a method that we employed earlier (Wu et al., 1977). ^{57}Co–Zn core RPase was oxidized by treatment with H_2O_2 to secure the Co ion at the prospective binding site in the enzyme. The oxidized enzyme was then denatured, and its subunits were separated by NaDodSO$_4$–polyacrylamide gel electrophoresis. Figure 1A shows that all ^{57}Co counts were associated with the β and/or β' subunits. The resolution of NaDodSO$_4$–polyacrylamide gel electrophoresis was not sufficient to assign the ^{57}Co counts to either the β or the β' subunit. For resolution of this problem, ^{57}Co(III)–Zn core RPase was denatured with 7 M urea and was chromatographed on an Affi-Gel Blue column which separates the $\alpha_2\beta$ subunit complex and the β' subunit. As can be seen from the chromatographic elution profile (Figure 2), the ^{57}Co counts were associated with the $\alpha_2\beta$ peak, but not with the β' peak. When the pooled fractions of the $\alpha_2\beta$ peak were electrophoresed on NaDodSO$_4$–polyacrylamide gel electrophoresis, the radioactivity profile showed that the ^{57}Co counts comigrated only with the β subunit (Figure 1B). These results indicated that the intrinsic Zn in the β subunit was replaced by Co ion. This conclusion was confirmed by atomic absorption spectrometry of the Co(III)–$\alpha_2\beta$ subunit complex

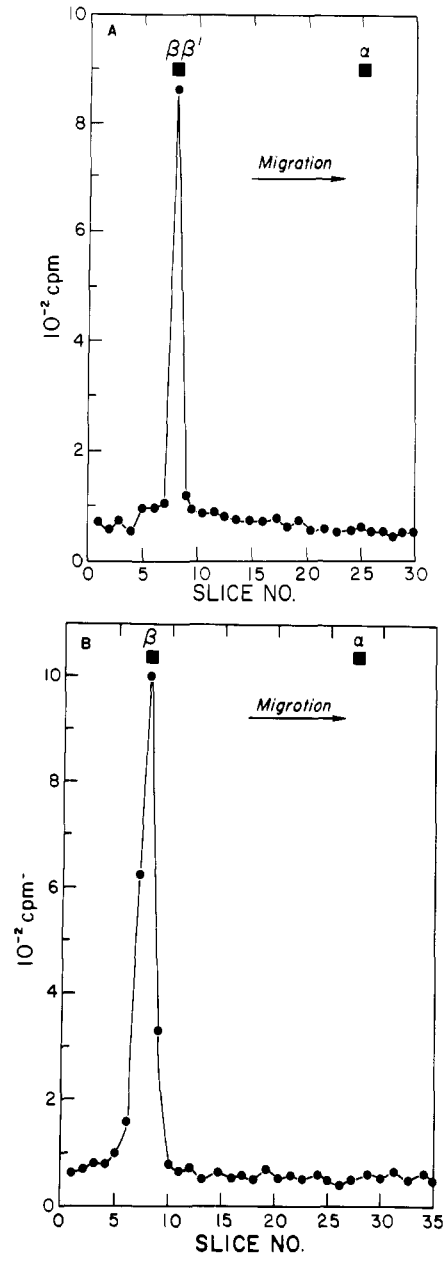

FIGURE 1: Radioactivity profiles of gel slices from NaDodSO$_4$–polyacrylamide gel electrophoresis of ^{57}Co(III)–core RPase (A) and ^{57}Co(III)–$\alpha_2\beta$ complex (B). 20 μg of ^{57}Co(III)–core RPase (4×10^3 cpm) or ^{57}Co(III)–$\alpha_2\beta$ complex (5×10^3 cpm) was loaded on a 7.5% acrylamide gel in the discontinuous buffer system according to the method of Laemmli (1970). After electrophoresis, thin slices (3 mm) of gel were cut, oxidized with 1 mL of 30% H_2O_2, and counted in an Isodyne Model 1185 automatic γ counter. ^{57}Co(III)–$\alpha_2\beta$ complex (2.5×10^4 cpm) obtained from the Affi-Gel Blue column (Figure 2) was concentrated by precipitation, redissolved in storage buffer, and mixed with 0.1 mL of cold $\alpha_2\beta$ complex before use for electrophoresis.

isolated by Affi-Gel Blue column chromatography (Table II).

Absorption Spectra of Metal Hybrid Enyzmes. Both Co–Zn and Ni–Zn core RPases possess characteristic absorption spectra in the near-UV and visible regions (Figure 3). The spectrum of the Co–Zn enzyme exhibits two major peaks at 400 nm (ϵ = 3000) and 475 nm (ϵ = 2700) and some minor bands near 580 and 700 nm. The spectrum of the Ni–Zn enzyme also has a major peak at 462 nm with a relatively large extinction coefficient (ϵ = 8000). The Cu–Zn RPase also has some weak absorption in the visible region. However, this was not studied further because of its low enzymatic activity. No visible absorption spectrum was detected for Mn–Zn RPase

Table II: Metal Contents of Native and Reconstituted Core RNA Polymerases and of the Subunit Complex Determined by Atomic Absorption Spectrometry[a]

enzyme	reconstitution	metal content (mol/mol of enzyme)				
		Zn	Co	Mn	Ni	Cu
core RPase	no	2.3 ± 0.1	<0.1	<0.1	<0.1	<0.1
	in Zn(II)	1.8 ± 0.2	<0.1	<0.1	<0.1	<0.1
	in Co(II)	1.1 ± 0.1	0.9 ± 0.2 (0.8 ± 0.1)[b]			
	in Mn(II)	1.2 ± 0.1		0.65 ± 0.05		
	in Ni(II)	1.2 ± 0.05			0.9 ± 0.1	
	in Cu(II)	1.1 ± 0.1				1.2 ± 0.1
Co(III)-$\alpha_2\beta$[c]	in Co(II)	0.1 ± 0.05	0.7 ± 0.1			

[a] The data represent the average of at least five enzyme preparations. [b] Determined by the radioactivity of ^{57}Co when core RPase was reconstituted in the presence of ^{57}Co(II). [c] Co(III)-$\alpha_2\beta$ was isolated from Co(II)-reconstituted core RPase by Affi-Gel Blue chromatography in the presence of 7 M urea after oxidation of bound Co(II) by H_2O_2.

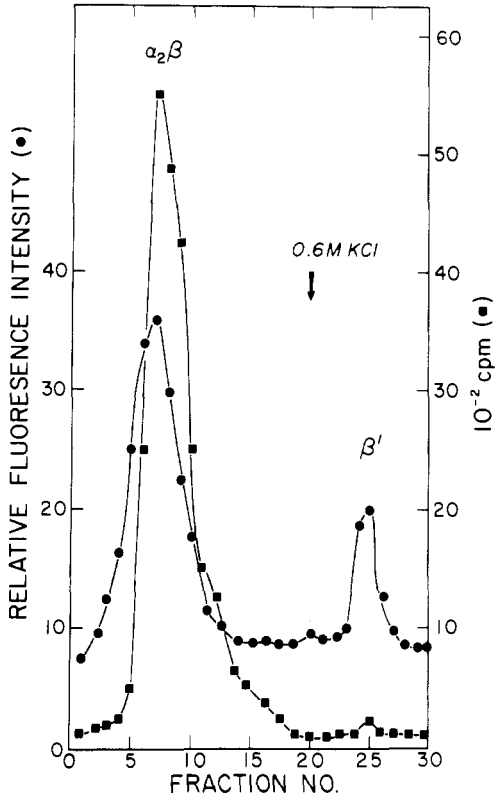

FIGURE 2: Typical elution profile of ^{57}Co(III)-core RPase from an Affi-Gel Blue column in the presence of 7 M urea. ^{57}Co-core RPase (4×10^4 cpm) was denatured by dialysis overnight at 4 °C against denaturation buffer [0.02 M Tris-HCl (pH 8), 0.01 M $MgCl_2$, 0.2 mM dithiothreitol, 10% glycerol, and 7 M urea]. The dialyzed enzyme was applied to an Affi-Gel Blue column (7 mL) which was equilibrated with the same buffer. Fractions (1 mL) were collected by eluting the column with the same buffer (peak for $\alpha_2\beta$) and the buffer + 0.6 M KCl (peak for the β' subunit). The protein peaks were located by measuring the fluorescence at 340 nm with an exciting wavelength of 280 nm.

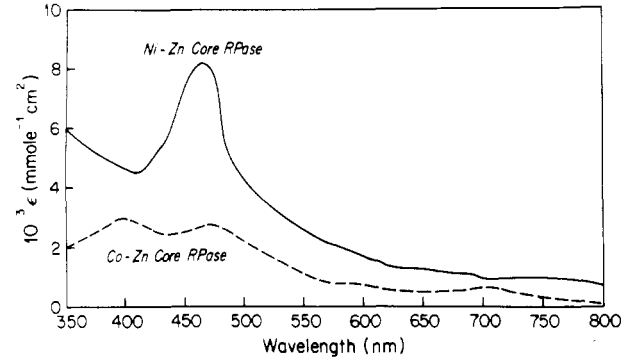

FIGURE 3: Visible and near-UV absorption spectra of Co–Zn (---) and Ni–Zn (—) core RPases. The sample was 2 mg/mL Co–Zn or Ni–Zn core enzyme in 0.05 M Tris-HCl (pH 8) containing 0.15 M KCl, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol. The reference was the same concentration of Zn–Zn core RPase in the same buffer. The spectrum was measured in a 0.5-mL masked quartz cuvette in a Cary 118 spectrometer with a scattering transmission accessory.

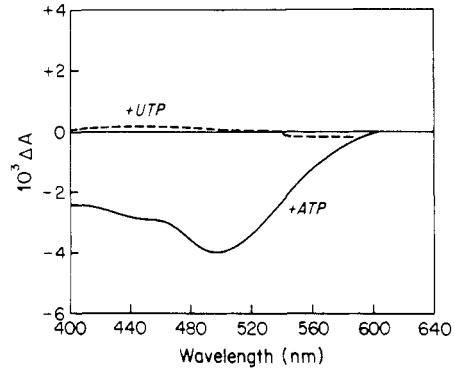

FIGURE 4: Effect of ATP and UTP on the visible spectrum of the Ni–Zn core RPase. Both the sample and reference cuvettes contained 2 mg/mL (5×10^{-6} M) Ni–Zn core RPase in 0.05 M Tris-HCl (pH 8), 0.15 M KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol. The difference spectrum was generated by the addition of 1 mM ATP (—) or UTP (---) to the sample cuvette. The dilution effect in the sample cuvette was compensated by adding an appropriate amount of buffer to the reference cuvette.

and for the $\alpha_2\beta$ complexes isolated from Ni–Zn and Co–Zn RPases.

The absorption spectrum of the Ni–Zn core enzyme could be perturbed by 1 mM ATP but not by the same concentration of UTP (Figure 4). Neither DNA template nor Mg(II) ion was required for such perturbation.

Other Comparative Studies. We have compared the metal hybrid enzymes with respect to their abilities for "abortive initiation" (Johnston & McClure, 1976). DNA from the T7 deletion mutant ΔD111 contains a single major promoter (A_1) with the initiation sequence AUCGA... (Studier, 1975). In the presence of only the first two nucleotides, ATP and UTP, this DNA will direct the steady-state synthesis of a dinucleoside tetraphosphate, pppApU (Oen et al., 1979). It was found that the activities of various metal hybrid enzymes to catalyze this reaction also decreased in the order Zn > Co > Mn > Ni > Cu. (The Cu-substituted enzyme was almost inactive.) However, kinetic analysis indicated that this order was primarily due to the difference in V_{max} (Table III). No significant difference was observed for the K_m of ATP and UTP for all metal hybrid enzymes.

Table III: Apparent K_m and V_{max} Values for ATP and UTP Obtained by Using T7 ΔD111 DNA as Template in the Abortive Initiation Assay[a]

RNA polymerase	K_m (mM)		V_{max} (nmol/h)	
	ATP	UTP	ATP	UTP
Zn-Zn	1.35	0.33	4.2	4.0
Co-Zn	1.18	0.31	2.9	3.2
Mn-Zn	1.11	0.28	2.5	2.8
Ni-Zn	1.25	0.29	2.0	2.0

[a] Kinetic constants were determined from double-reciprocal plots of the initial velocity data. For determinations of the K_m and V_{max} values of ATP and UTP, the UTP and ATP concentrations were held at 0.2 and 2 mM, respectively.

We have also examined the fidelity of transcription carried out by various metal hybrid enzymes. This was determined by the incorporation ratio of noncomplementary ([^{3}H]GMP or CMP) to complementary ([α-^{32}P]AMP or UMP) nucleotide into RNA synthesized on poly(dA-dT) template (Springgate & Loeb, 1975). As found for Co–Co RPase (Speckhard et al., 1977), no significant difference was observed for the fidelity of transcription among all metal hybrid enzymes. These metal hybrid enzymes, resembling native RPase, were unable to utilize deoxynucleoside triphosphate as substrate.

Discussion

In this study, we have developed a sequential denaturation–reconstitution method to substitute in vitro one of the two intrinsic Zn ions in *E. coli* RPase with other divalent metals. This in vitro substitution method is simpler than the elaborated in vivo substitution procedure reported earlier (Speckhard et al., 1977). In addition, the in vivo procedure substitutes both Zn ions by biosynthetic incorporation, whereas the in vitro method selectively replaces one of them. Presumably, the conditions used for denaturation (7 M urea) allowed the removal of only one of the two Zn ions in the enzyme, which was then substituted by another divalent metal ion added during the reconstitution process. Using this method, we have successfully replaced one Zn ion in *E. coli* RPase with Co, Mn, Ni, or Cu. Similar substitution can, in principle, be carried out with other divalent metals (e.g., Cd). Since the bound Zn ions in multisubunit RPases are not readily removed or exchanged with other metal ions in vitro (Wu & Wu, 1981), the denaturation–reconstitution method developed here may also be employed in the substitution of intrinsic metals in other RPases.

The substituted metal in Co–Zn RPase was found to be in the β subunit. This was identified by determination of the metal content in individual subunits or the subunit complex separated under denaturation conditions. Co–Zn RPase was first oxidized by H_2O_2 in order to secure the metal ion at its prospective binding site in the enzyme under denaturation conditions. The oxidation transformed Co(II) from the exchange-labile (d^7) to the exchange-inert (d^6) Co(III) state and therefore offered a method to "freeze" Co ion at its binding site. This method has been applied to other Co-substituted enzymes, such as carbonic anhydrase (Lindskog, 1970), carboxypeptidase (Coleman & Vallee, 1960), and alkaline phosphatase (Applebury & Coleman, 1969). Assumption was made in these studies that the oxidation did not change the location of the metal in the enzyme.

In an earlier study, the Zn content was found to be 1.4 ± 0.5 mol/mol of protein for the β' subunit and 0.6 ± 0.3 mol/mol of protein for the β subunit (Wu et al., 1977). The nonintegral stoichiometry was interpreted as suggesting that at least one of the two tightly bound Zn ions in RPase is located in the β' subunit, while the other Zn ion may be in β' or β, or at the contact domain of these two subunits. This ambiguity was eliminated by better data obtained later which indicated that the two ions were evenly distributed between β and β'. A similar result was also reported by Miller et al. (1979). The finding that only the intrinsic Zn ion in the β subunit was substituted by the denaturation–reconstitution method further confirms the stoichiometric distribution of Zn ions between β and β' and is consistent with our previous observation that the intrinsic Zn ion located in the β' subunit could not be removed from the enzyme by 7 M urea under similar denaturation conditions (Wu et al., 1977).

Although reconstitution of RPase with high yield (>90%) from its denatured state has been mentioned (Ishihama & Ito, 1972; Zillig et al., 1970), under the experimental conditions described in this paper, we consistently recovered only 50–70% of the initial enzyme activity. Since a significant fraction of the enzyme remains denatured in some way, one may question whether the Co–Zn enzyme system represents a homogeneous species or a mixture consisting of Co–Co and Zn–Zn enzyme molecules. Several lines of evidence support the contention that the Co–Zn RPase system is predominantly a homogeneous species with one Co ion in the β subunit and one Zn ion in the β' subunit of the same enzyme molecule. First, because the two intrinsic metal ions in RPase are evenly distributed between β and β' subunits, the absence of Co in the β' subunit (Figure 2) tends to rule out the possible existence of the Co–Co enzyme. Second, the $\alpha_2\beta$ complex isolated from Co–Zn RPase contains approximately one Co ion with a negligible amount of Zn (Table II), again indicating that the Zn–Zn enzyme is not a significant constituent of the Co–Zn enzyme system. Finally, although the reconstituted Co–Zn enzyme recovered less than 100% of enzyme activity as compared to native enzyme, it is as active as the similarly reconstituted Zn–Zn enzyme, suggesting that the reduction in enzyme activity is not due to the metal substitution or redistribution. In addition, that the reconstituted Co–Zn, Mn–Zn, Ni–Zn, and Cu–Zn RPases possess 100, 100, 60, and 17% of the enzyme activity as compared to the reconstituted Zn–Zn enzyme indicates that Co and Mn are the best substitutes for Zn. The high activity of Co–Zn RPase was not surprising because Co–Co RPase obtained biosynthetically was as active enzymatically as the Zn–Zn enzyme (Speckhard et al., 1977). Moreover, the low activity of Cu–Zn RPase may give an explanation for our previous observation (Speckhard et al., 1977) that *E. coli* cells do not grow well in the Zn-depleted, Cu-enriched medium. It should be pointed out, however, that the preparations of metal hybrid RPases may still contain a small fraction of Zn–Zn enzyme due to incomplete removal of Zn ions in the reconstitution buffer. Thus, the specific activities of metal hybrid enzymes listed in Table I may not be precise but represent only the upper limits of the actual values. Nevertheless, this limitation would not alter the conclusion that the enzymatic activities of metal-substituted RPases decrease in the order Zn = Co = Mn > Ni > Cu.

A similar order of activities was also observed for abortive initiation (Table III), suggesting that the variation in the enzymatic activities of metal-substituted RPases resides at the level of RNA chain initiation. It has been suggested (Scrutton et al., 1971) that the intrinsic metal of RPase may play a role in initiation. With T7 DNA as template, the ratio of RNA chains starting with GTP to those starting with ATP was less for Co–Co RPase than for Zn–Zn RPase (Speckhard et al., 1977). One possible interpretation of this observation is that the intrinsic metal ion may be located at the initiation site on

the enzyme, thereby discriminating the initiating nucleotides. Since the substituted metal is located in the β subunit which contains the initiation site, the metal hybrid RPases may be used to test this possibility. We have, therefore, carried out the steady-state kinetic analysis of the abortive initiation reaction directed by T7 ΔD111 DNA. To our disappointment, K_m values for ATP, which may reflect the affinity of this nucleotide for the initiation site, did not vary significantly among various metal hybrid enzymes. However, the interpretation of these results may be complicated by the fact that there is no conclusive evidence which indicates that abortive initiation and productive initiation of RNA synthesis actually go through the same kinetic pathway and that they may have different rate-limiting steps (Shimamoto et al., 1981). In addition to the kinetic parameters for abortive initiation, all metal hybrid RPases did not exhibit significant differences in their abilities to incorporate noncomplementary (fidelity of transcription) or erroneous (deoxyribonucleotide) nucleotides into RNA product.

More clear-cut evidence which shows that the intrinsic Zn in the β subunit may be involved in binding the initiating nucleotide comes from the absorption spectroscopic studies. Replacement of this Zn with Co or Ni resulted in the formation of intense absorption bands (ϵ = 3000–8000) in the 400–500-nm range which may be attributable to charge-transfer transitions (Day & Jorgensen, 1964). A similarly intense charge-transfer band was also reported for nickel aspartate transcarbamylase (Johnson & Schachman, 1980). The charge-transfer bands were not observed previously for Co–Co RPase (Speckhard et al., 1977) because of some technical limitations. The high concentration (10 mg/mL) of enzyme solution used in the difference absorption spectroscopy produced a serious light scattering problem which made the peak assignment in the far-UV region rather difficult. This problem was partially resolved by use of a powerful scattering transmission device in the present study. The charge-transfer bands are more intense than the d–d transition bands which occur in the longer wavelength region (500–700 nm). The latter absorption bands which were first observed for Co–Co RPase derived from in vivo incorporation (Speckhard et al., 1977) could also be detected with Co–Zn enzyme obtained by in vitro substitution. This suggests that the Co ion in the β subunit of these two species possesses a common irregular coordination in a nearly tetrahedral environment (Lindskog, 1970). Such an environment does not exist in the $\alpha_2\beta$ complex isolated from Co–Zn or Ni–Zn RPase which exhibits no visible absorption band. Also, it was suggested previously (Speckhard et al., 1977) that the 584- and 703-nm peaks for Co–Co RPase might arise from different Co ions in the enzyme. The detection of both those peaks in the Co–Zn enzyme clearly indicated that this is not the case. The most interesting finding is that the visible absorption spectra of Co–Zn or Ni–Zn RPase could be perturbed by addition of nucleoside triphosphate. It was shown that there are two nucleoside triphosphate binding sites on *E. coli* RPase, the initiation site and the elongation site (Wu & Goldthwait, 1969). The initiation site binds primarily purine nucleotides in the absence of template. Mg(II) ion is required for binding of a nucleotide to the elongation site but not to the initiation site. The fact that perturbation of the visible spectrum of Ni–Zn RPase by ATP does not require the presence of Mg(II) ion or DNA template is consistent with the contention that the substituted metal is located at the initiation site. UTP, which is not a purine nucleotide and has low affinity for the initiation site, did not alter the absorption spectrum. However, the interpretation of the absorption studies may be complicated by the question raised before of whether these spectral changes are due to a direct or indirect effect of the nucleotide binding to the intrinsic metal at the initiation site on the enzyme . An NMR spectroscopic study to resolve this problem will be described in the following paper (Chatterji & Wu, 1982).

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