Role of the Intrinsic Metal in RNA Polymerase from *Escherichia coli*. In Vivo Substitution of Tightly Bound Zinc with Cobalt†

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ABSTRACT: *Escherichia coli* RNA polymerase is a metalloenzyme containing 2 g-atoms of tightly bound zinc per mol of enzyme. We have prepared RNA polymerase from *E. coli* cells grown in a zinc-depleted medium supplemented with cobalt(II) chloride. The purified enzyme contains 1.8–~2.2 g-atoms of cobalt per mol of enzyme with concomitant reduction in the zinc content. The cobalt-substituted enzyme is enzymatically as active as Zn-RNA polymerase on a variety of templates under standard assay conditions. These two enzymes are almost identical by such physical criteria as subunit composition, monomer–dimer equilibrium, and pH and temperature stabilities. They differ in that Co-RNA polymerase exhibits a visible absorption spectrum with two major peaks at 584 and 703 nm. Addition of nucleoside triphosphates selectively perturbs the 584-nm peak, whereas addition of a template analogue, $d(pT)_{10}$, affects both peaks. These spectral changes suggest that the tightly bound metal ions may directly or indirectly participate in binding of substrate or template to the enzyme. Biochemically, both enzymes are also very similar with respect to pH–activity profile, extrinsic metal requirements, 1,10-phenanthroline inhibition, and fidelity of transcription of synthetic templates. Detailed kinetic and biochemical analyses have revealed that the Co enzyme has a lower value (~twofold) of apparent K_m for T7 DNA under certain experimental conditions and that it is less efficient in initiating RNA chains at the A_2 than at the $A_1 + A_3$ promoters on T7 DNA template as compared to the Zn enzyme. This has been demonstrated by studying the ratio of GTP/ATP incorporations into the 5′ terminal of RNA products and by measuring the formation of $(rI)_n$-resistant initiation complexes at specific promoter sites using various combinations of dinucleotides and nucleoside triphosphates. Moreover, the in vitro transcription of a *lac* operon system by Co-RNA polymerase is less sensitive to cAMP and cAMP receptor protein than is the transcription by Zn-RNA polymerase. The results of our comparative studies using the Co and Zn enzymes further support the contention that the intrinsic metal of RNA polymerase is involved in promoter recognition and specific initiation in RNA synthesis.

A variety of nucleotidyl transferases including DNA and RNA polymerases from both prokaryotic and eukaryotic sources have been shown to be zinc metalloenzymes (Slater et al., 1971; Scrutton et al., 1971; Auld et al., 1974; Coleman, 1974). The DNA-dependent RNA polymerase (EC 2.7.7.6) purified from *Escherichia coli* contains 2 g-atoms of tightly bound zinc per mol of enzyme (Scrutton et al., 1971). These zinc ions cannot be removed from the enzyme by chelating reagents such as EDTA or 1,10-phenanthroline. 1,10-Phenanthroline is an inhibitor of RNA chain initiation. This effect is probably due to the ability of the inhibitor to chelate metal ions and not to a nonspecific interaction with a hydrophobic region of the enzyme, since the nonchelating analogue, 1,7-phenanthroline, is a less effective inhibitor and shows a different pattern of inhibition. Thus, there is indirect evidence for a role of the bound zinc in the initiation of RNA synthesis.

The role of the bound divalent metal in transcription might be rendered more amenable to investigation if the zinc could be replaced with another metal, e.g. cobalt or manganese, which unlike zinc would be expected to possess optical and magnetic properties sensitive to agents that can affect the enzymatic activity. Such a method has been applied successfully to a number of Zn-metalloenzymes (Lindskog, 1970). In this communication, we report the substitution of zinc in *E. coli* RNA polymerase with cobalt.

Materials and Methods

Materials. Metal standards and cobalt(II) chloride were obtained from Fischer Scientific Co., Chelex-100 from Bio-Rad, and 1,10- and 1,7-phenanthroline from K&K Rare and Fine Chemicals. Unlabeled nucleoside triphosphates and $d(pT)_{10}$[1] were obtained from P-L Biochemicals. ^{3}H-Labeled ribonucleoside triphosphates and ^{32}P-labeled sodium pyrophosphate were from New England Nuclear Corp. α- and γ-^{32}P-labeled ATP and GTP were purchased from ICN Pharmaceutical Co. Poly[d(A-T)] and calf-thymus DNA were products of GIBCO and Worthington, respectively. Cyclic AMP, dinucleotides, and poly(riboinosinic acid) $[(rI)_n]$ were purchased from Sigma. T7 DNA was prepared as described by Sadowski (1971). The 796 base pair, *Hin*dII + -III restriction fragment of λ*plac5* DNA (*lac*-796) was prepared by Z. Hillel and P. Bandyopadhyay. Cyclic AMP receptor protein (CRP) was purified from *E. coli* as described previously (Wu et al., 1974).

Cell Growth. The standard minimal medium M63 contained

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[1] Abbreviations used are: RPase or Zn-RPase, RNA polymerase; Co-RPase, cobalt-substituted RNA polymerase; $(rI)_n$, poly(riboinosinic acid); EDTA, ethylenediaminetetraacetic acid; *lac*-796, the 796 base pair, *Hin*dII + -III restriction fragment of λ*plac5* DNA; cAMP, cyclic adenosine 3′,5′-monophosphate; CRP, cAMP receptor protein; NTP, nucleoside triphosphate; CpC, cytidylyl-(3′→5′)-cytidine; CpG, cytidylyl-(3′→5′)-guanosine; ApC, adenylyl-(3′→5′)-cytidine; $d(pT)_{10}$, oligo(deoxythymidylic acid) with 10 deoxythymidylic acid residues.

2 g of $(NH_4)_2SO_4$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, 1 mL of 1 M $MgSO_4$, 2 mL of glycerol, and 100 mL of 1 M phosphate buffer (pH 7) per liter. To reduce the amount of Zn^{2+} in the medium, the phosphate buffer was passed through a Chelex-100 column. The Co^{2+} supplementation was achieved by addition of $CoCl_2$ to a final concentration of 5×10^{-6} M. The glass carboys used to grow the cells were soaked in 30% nitric acid for 2–3 days and then washed with deionized, distilled H_2O. The cells were grown to essentially stationary phase at 37 °C and harvested with a Sorvall continuous flow centrifuge.

Enzyme Purification. RNA polymerase was purified by a procedure developed in our laboratory (Wu and Wu, 1973) and by the method of Burgess and Jendrisak (1975) with the following precautions: all glassware was acid washed and rinsed with deionized, distilled H_2O, all reagents were the highest purity available and when necessary treated with Chelex-100, and all buffers contained 1 mM EDTA. The enzyme preparations used for physical and biochemical studies were further purified by glycerol gradient centrifugation. These preparations were at least 98% pure as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were essentially free of DNase, RNase, ATPase, and GTPase. The yield of RNA polymerase isolated was about two- to threefold lower from *E. coli* cells grown with cobalt than without cobalt.

Biochemical Assays. RNA polymerase activity was assayed by the incorporation of ^3H-labeled ribonucleoside monophosphate into acid-insoluble material as described previously (Wu and Wu, 1973). The standard reaction mixture (0.25 mL) contained 0.08 M Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 0.4 mM each of ATP, GTP, CTP, and UTP (one labeled with ^3H, $\sim 5 \times 10^3$ cpm/nmol), 0.03 μmol of calf-thymus (or T7) DNA, and 2–5 μg of the enzyme. When poly[d(A-T)] was used as template, GTP and CTP were omitted and 0.2 M KCl was added to the reaction mixture. The incubation was for 10 min at 37 °C. The reaction was stopped by addition of 0.1 mL of 0.1 M sodium pyrophosphate and 5 mL of cold 5% trichloroacetic acid. The acid-insoluble material was collected on a glass-fiber filter (Whatman GF/C, 2.4 cm) and washed with cold 1% trichloroacetic acid and ethanol. The radioactivity was measured in a liquid scintillation counter.

The binding of RNA polymerase to [^3H]T7 DNA was assayed by retention of the binary complex on a nitrocellulose membrane using the modified procedure of Hinkle and Chamberlin (1972). The procedure used for measuring the DNA-dependent [^{32}P]pyrophosphate exchange catalyzed by RNA polymerase was essentially as described by Krakow and Fronk (1969). The assay of RNA chain initiation by the incorporation of γ-^{32}P-labeled ATP or GTP into the acid-insoluble material was carried out according to Maitra and Hurwitz (1965) with slight modification. For the $(rI)_n$-resistant RNA synthesis with various dinucleotides and nucleotide triphosphates, the method of Dausse et al. (1975) was used. To analyze the fidelity of transcription by RNA polymerase, we measured the incorporation of complementary ribonucleotide (AMP or UMP) and noncomplementary ribonucleotide (CMP or GMP) with poly[d(A-T)] template following the procedure of Springgate and Loeb (1975).

Transcription of restriction fragment *lac*-796 of $\lambda plac5$ DNA by Co- and Zn-RNA polymerase holoenzyme was performed as follows. The reaction solution (0.125 mL) contained 0.02 M Tris-HCl (pH 8), 0.05 M KCl, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.3 mM dithiothreitol, 0.4 mM each of GTP, CTP, UTP, and [^3H]ATP (14 000 cpm/nmol), 0.01 A_{260} unit of *lac*-796 fragment, and 5.6 μg of enzyme. Where indicated, 7 μg of CRP and 0.1 mM cAMP were added. Samples transcribed in the presence of CRP and cAMP were preincubated for 10 min at 37 °C without enzyme. Reaction proceeded for 45 min at 37 °C and was stopped by placing solutions on ice. Sodium pyrophosphate (0.2 M; 25 μL) was added and RNA product was coprecipitated with 0.1 A_{260} unit of $\lambda plac$ DNA by adding 5 mL of 5% trichloroacetic acid. Precipitates were filtered on nitrocellulose disks and washed with 1% trichloroacetic acid, and the radioactivity was counted in a scintillation counter.

The protein concentration was determined by the method of Bücher (1947) or the method of Lowry et al. (1951) as standardized by Berg et al. (1971) for RNA polymerase.

Metal Determination. Metal concentrations were determined using a Perkin-Elmer atomic absorption spectrophotometer Model 360, equipped with a carbon arc furnace HGA 2100. RNA polymerase (0.1–~1 mg/mL) was dialyzed at least 16 h against 0.01 M Tris-HCl buffer (pH 8) containing 10 mM EDTA, 5% glycerol, 0.1 M KCl, and 0.1 mM dithiothreitol, and was diluted 0- to ~20-fold with deionized, distilled H_2O before determination of its metal content. Aliquots, 5–10 μL, of the samples were used for Zn determination, and 10–40 μL was for Co determination. Metal standard solutions were prepared by dilution of the Fisher Standard for zinc and cobalt in 0.01 N HNO_3. Metal analysis was performed on all preparations utilized for the activity and spectral measurements.

Results

Biosynthetic Incorporation of Cobalt into RNA Polymerase. A direct way to achieve metal substitution in metalloenzyme is to remove the constitutive metal and replace it with another metal in vitro. For RNA polymerase, the in vitro substitution was difficult because of the extremely tight binding of zinc to the enzyme. The zinc could not be removed by 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 caused a loss of zinc and a concomitant inactivation of the enzyme not reversible by addition of zinc. Furthermore, the tightly bound zinc could not be exchanged with either Co^{2+}, Cd^{2+}, Cu^{2+}, or Mn^{2+} present in the dialysis solution. Therefore, we have attempted to achieve substitution of Co^{2+} for Zn^{2+} in RNA polymerase by incorporation in vivo.

Figure 1 shows the growth curves of *Escherichia coli* K12 in minimal media with various metal contents at 37 °C. The standard minimal medium (M63) contained about 5×10^{-6} M Zn^{2+}, which could be reduced to about 5×10^{-8} M by treating with Chelex-100. As can be seen in the figure, the bacterial cells grew normally in the Zn^{2+}-depleted medium. Addition of 5×10^{-6} M Co^{2+} to the Zn^{2+}-depleted medium caused a lag period of 2–3 h after which growth resumed. The final cell density was the same as with the standard or Zn-depleted minimal medium. In contrast, relatively little growth was observed in the Zn^{2+}-depleted medium supplemented with 5×10^{-6} M of Cu^{2+}.

RNA polymerase was purified by the standard procedure (Wu and Wu, 1973; Burgess and Jendrisak, 1975) from *E. coli* cells grown in the Co^{2+}-enriched, Zn^{2+}-depleted medium. In all preparations, Co^{2+} copurified with the enzyme. When the purified enzyme was eluted with a salt gradient on a DE-52 column, the elution profiles of Co^{2+} and enzyme activity coincided, while the Zn^{2+} concentration was quite low throughout the protein peak. This is demonstrated in Figure 2. If Co^{2+} were bound to some protein impurities, the Co^{2+} content and the enzyme activity would not coincide. Thus the

TABLE I: Metal Contents and Specific Activity of RNA Polymerase from *E. coli* Grown in Standard and Cobalt-Enriched Zinc-Depleted Media.

Media	Strain	Zn content (g-atoms/mol)	Co content (g-atoms/mol)	Zn + Co (g-atoms/mol)	% Co	Sp act. (units[a]/mg)
Standard	MRE600	2.0 ± 0.1	0	2.0	0	1150
	MRE600	2.1 ± 0.2	0	2.1	0	930
	K12	2.2 ± 0.3	0	2.2	0	880
	K12	1.9 ± 0.2	0	1.9	0	1020
	Average	2.1 ± 0.2	0	2.1 ± 0.2	0	995 ± 118
Co-enriched	MRE600	0.1 ± 0.1	2.2 ± 0.3	2.3	95	1095
+	MRE600	0.2 ± 0.1	1.8 ± 0.2	2.0	90	1105
Zn-depleted	K12	0.2 ± 0.1	1.8 ± 0.3	2.0	90	990
	K12	0.2 ± 0.1	2.1 ± 0.1	2.3	91	1180
	Average	0.2 ± 0.1	1.9 ± 0.2	2.1 ± 0.2	92 ± 2	1043 ± 166

[a] One unit is defined as the nmol of radioactive-labeled nucleoside monophosphate incorporated in 20 min at 37 °C.

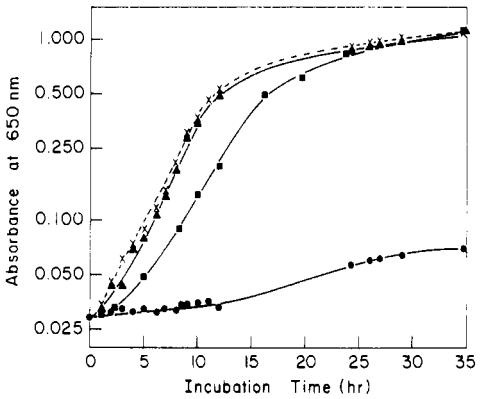

FIGURE 1: Growth curves of *Escherichia coli* K12 at 37 °C in media with various metal contents: (×) the standard minimal medium (M63) containing 5×10^{-6} M Zn^{2+}; (▲) the Chelex-100 treated minimal medium (Zn depleted); (■) the Chelex-100 treated minimal medium to which 5×10^{-6} M Co^{2+} was added; (●) the Chelex-100 treated minimal medium to which 5×10^{-6} M Cu^{2+} was added.

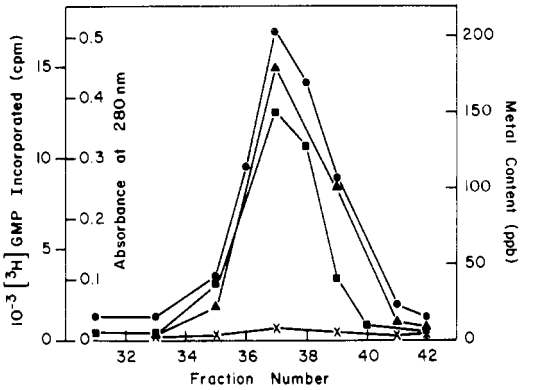

FIGURE 2: DE-52 chromatography of Co-RNA polymerase. Co-RNA polymerase (4 mg) was dialyzed against buffer I (0.01 M Tris-HCl (pH 8.0), 0.01 M $MgCl_2$, 0.1 M KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol) and the dialyzed enzyme was applied to a 9 cm × 1 cm^2 DE-52 column preequilibrated with the same buffer. The column was washed with buffer I + 0.14 M KCl and then eluted with a salt gradient of 0.14–0.23 M KCl in buffer I. Fractions of 0.6 mL were collected for metal analysis, enzyme assay, and protein determination: (×) Zn^{2+} content; (■) Co^{2+} content; (▲) RNA polymerase activity; and (●) absorbance at 280 nm.

Co^{2+} was bound to RNA polymerase. In addition, the binding was very tight. The bound Co^{2+} could not be removed by dialysis for 24 h at 4 °C against 10 mM EDTA in Tris-HCl buffer (pH 8) or by treatment with Chelex-100.

An analysis of the metal contents of RNA polymerase preparations purified from *E. coli* K12 and MRE600 grown in the standard and Co^{2+}-enriched, Zn^{2+}-depleted media is presented in Table I. RNA polymerase preparations from cells grown in the standard growth medium contain about 2 g-atoms of Zn^{2+} per mol of enzyme without any detectable amount of Co^{2+} present. In enzyme preparations isolated from cells grown in the Co^{2+}-enriched, Zn^{2+}-depleted medium, the Co^{2+} content of the enzyme varies from 1.8 to 2.2 g-atoms per mol of enzyme, and the Zn^{2+} content is less than 0.3 g-atom per mol of enzyme. The total (Co^{2+} + Zn^{2+}) content is constant (2.1 ± 0.2 g-atoms per mol of enzyme) and is independent of the bacterial strain or the type of medium used. Thus, through biosynthetic incorporation, we have successfully replaced more than 90% of the intrinsic Zn^{2+} in RNA polymerase with Co^{2+}.

Physical Properties of Co-Substituted RNA Polymerase. Zn- and Co-RNA polymerases are physically almost identical. Both enzymes gave the same band pattern (2α, β, β', and σ) by the polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Burgess, 1969). When analyzed by zonal sedimentation in glycerol gradients, Co-RNA polymerase existed as monomer at a high salt concentration (0.4 M KCl) and dimerized at a low salt concentration (0.05 M KCl), similar to what has been observed for Zn enzyme (Berg and Chamberlin, 1970). No significant difference between these two enzymes was observed in pH and temperature stability profiles.

All preparations of Co-RNA polymerase show a characteristic absorption spectrum in the visible region. Figure 3 shows the difference absorption spectra between Co-RNA polymerase and Zn-RNA polymerase in the visible region. There are two major peaks with maxima at 584 and 703 nm, and a minor peak near 750 nm. None of the Zn-RNA polymerase preparations had absorption bands in this region. The absorption spectrum of Co enzyme could be altered by addition of substrates or template. ATP or GTP selectively perturbed the 703-nm peak without apparent change in the 584-nm peak. UTP or CTP showed a similar but smaller effect. Addition of a template analogue, $d(pT)_{10}$, reduced the amplitudes of both 703- and 584-nm peaks and slightly shifted their maxima. The spectral changes induced by nucleoside triphosphate and $d(pT)_{10}$ appeared to be additive.

Biochemical Properties of Co-Substituted RNA Polymerase. Co-RNA polymerase is enzymatically as active as the Zn-enzyme (Table I). Using various templates (e.g., calf-thymus DNA, T7 DNA, poly[d(A-T)], etc.), the specific activities of Co- and Zn-enzymes are essentially the same under

TABLE II: Initiation of RNA Chains with T7 DNA as Template.[a]

[KCl] (M)	Enzyme	[γ-^{32}P]ATP incorp. (pmol)	[γ-^{32}P]GTP incorp. (pmol)	A/G ratio	(A + G) starts/enzyme
0.05	Co-RPase	3.16	0.85	3.7	0.53
	Zn-RPase	3.08	1.16	2.6	0.54
0.20	Co-RPase	2.83	0.58	4.9	0.39
	Zn-RPase	2.24	1.07	2.1	0.34
0.25	Co-RPase	0.79	0.14	5.6	0.16
	Zn-RPase	0.50	0.28	1.8	0.13

[a] [γ-^{32}P]ATP and [γ-^{32}P]GTP incorporations were measured in reaction mixtures (0.1 mL) containing 0.05 M Tris-HCl (pH 8), 0.05–0.25 M KCl as indicated, 0.01 M $MgCl_2$, 5 mM dithiothreitol, 0.6 A_{260} unit of T7 DNA, 0.4 mM each of GTP (or ATP), CTP, and UTP, 0.4 mM [γ-^{32}P]ATP (or GTP) (50 cpm/pmol), and 3 to 5 μg of enzyme. The incubation was at 37 °C for 2 min; the reaction was then quenched by addition of 0.2 mL of 0.2 M sodium pyrophosphate, 0.02 mL of 50 mM ATP (or GTP), 0.1 mL of 5 mg/mL bovine serum albumin, and 1 mL of 3.5% $HClO_4$ in 0.1 M sodium pyrophosphate. After 5 min at 0 °C, the reaction mixture was centrifuged and the supernatant discarded. The pellet was resuspended in the above quenching solution containing 0.3 mL of 0.2 N NH_4OH. The centrifugation and suspension of the pellet were repeated and the final precipitates were collected on a GF/C filter presoaked in 3.5% $HClO_4$ in 0.1 M sodium pyrophosphate. The filter was washed with 30–40 mL of 3.5% $HClO_4$ in 0.1 M sodium pyrophosphate before drying with ethanol and counting in Econofluor in the Beckman liquid scintillation counter.

standard assay conditions. The pH-activity profile and the extrinsic metal requirements both for monovalent cations (K^+, Na^+, NH_4^+) and for divalent cations (Mg^{2+}, Mn^{2+}) appeared to be similar for these two enzymes. Moreover, like the Zn-enzyme, Co-RNA polymerase was inhibited by the metal chelator 1,10-phenanthroline (1 mM). EDTA is a much less effective inhibitor for both enzymes even at a tenfold higher concentration. Another similarity between Co- and Zn-RNA polymerase is their fidelity in gene transcription. The accuracy of transcription by RNA polymerase on poly[d(A-T)] template was determined by the ratio of noncomplementary ([^{3}H]CMP or GMP) to complementary ([α-^{32}P]UMP or AMP) bases incorporated into RNA product. Our results indicate that this ratio was about 1:3000 for CMP and about 1:40 000 for GMP for both Zn- and Co-RNA polymerase. These values are in agreement with that reported by Springgate and Loeb (1975) for Zn-enzyme.

Are there any differences between the biochemical properties of Co- and Zn-RNA polymerase? One subtle difference became evident upon closer kinetic studies. By steady-state kinetic analysis, we have found that V_{max} was the same for both Zn- and Co-enzymes as expected from the specific activities shown in Table I. The apparent K_ms for UTP and ATP were also similar ($\sim 5 \times 10^{-5}$ M) for both enzymes when poly[d(A-T)] was used as template. On the other hand, the apparent K_m for T7 DNA was lower (about a factor of two) with Co-enzyme at 0.2 M salt (KCl) concentrations. This difference was not observed with less specific DNA templates (calf-thymus DNA or poly[d(A-T)]) or at lower salt concentrations (<0.1 M KCl).

Role of Intrinsic Metal in RNA Chain Initiation. We have attempted to study the role of intrinsic metal in RNA chain initiation by direct comparison of the RNA chain initiations carried out by Zn- and Co-RNA polymerase. When the DNA-dependent pyrophosphate exchange reaction (Krakow and Fronk, 1969) was used as an indication of RNA chain initiation, no significant difference was detected. RNA chain initiation can also be studied by measuring the incorporation of γ-^{32}P-labeled nucleoside triphosphates into the 5′-terminal position of RNA product (Maitra and Hurwitz, 1965). Using T7 DNA as template, both Co- and Zn-RNA polymerase initiated about the same number of RNA chains as measured by γ-^{32}P-labeled nucleotide incorporations, but the Co-enzyme was less efficient in starting the RNA chains with GTP (Table II). This difference is best shown by the ratio of A starts to G starts. For the zinc enzyme, this ratio was about 2 in accord with a value reported by Chamberlin and Ring (1972). For the Co-enzyme, the ratio was 3.7–5.6 depending on the salt concentration. As shown in Table II, increasing salt concentrations increased the ratio of A starts to G starts with Co-RNA polymerase. In contrast, the ratio decreased with Zn-RNA polymerase. In both cases, the total RNA chain initiated per enzyme molecule decreased with increasing salt concentrations.

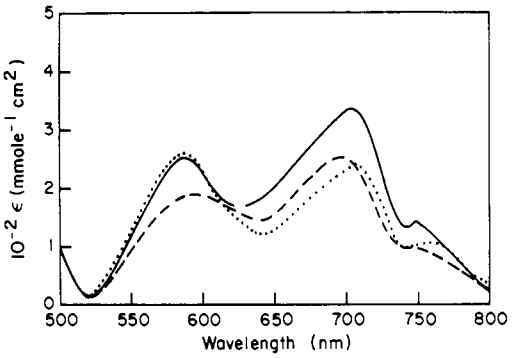

FIGURE 3: Difference spectra between Co- and Zn-RNA polymerase. The sample was 10 mg/mL Co-RNA polymerase in 0.05 M Tris-HCl buffer (pH 8) containing 0.05 M KCl, 0.01 M $MgCl_2$, 5% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol. The reference was the same concentration of Zn-RNA polymerase in the same buffer. The spectrum was measured in a 0.5-mL masked quartz cuvette using a Cary 118C spectrophotometer equipped with a scatter-transmission accessory. Both the sample and reference cells were thermostated at 15 °C: (—) no substrate or template added; (···) 0.4 mM ATP or GTP added; (- - -) 10^{-5} M $d(pT)_{10}$ added. The molar absorptivity was calculated from the measured absorbance with a path length of 1 cm and the molar concentration of cobalt ion.

Another way to study the effect of Co^{2+} substitution on the initiation of RNA chains by the enzyme on T7 DNA is to use a method devised by Dausse et al. (1975) for directing RNA polymerase to a single promoter site on T7 DNA. These authors showed that certain combinations of a dinucleotide primer and a nucleoside triphosphate can stabilize the enzyme on the DNA template at specific promoter sites by the formation of ternary initiation complexes. These complexes are resistant to inhibition by $(rI)_n$ at 0 °C. They were able to select the three major promoter sites on T7 DNA by using the following combinations: ApC + ATP selected promoter A_3, CpG + CTP selected promoter A_2, and CpC + ATP preferentially

TABLE III: Formation of Initiation Complexes at Specific Promotor Sites on T7 DNA Measured by $(rI)_n$-Resistant RNA Synthesis after Preincubation with Various Dinucleotides and Nucleoside Triphosphates.[a]

Enzyme	[KCl] (M)	% $(rI)_n$-resistant RNA synthesis: A_1 (CpC + ATP)	A_2 (CpG + CTP)	A_3 (ApC + ATP)	$(A_1 + A_3)/A_2$
Zn-RPase	0.05	35	37	31	1.8
	0.2	14	19	19	2.0
Co-RPase	0.05	44	24	30	3.1
	0.2	15	12	30	4.2

[a] Standard reaction mixture (0.2 mL) contained 0.04 M Tris-HCl (pH 7.9), 0.01 M $MgCl_2$, 0.1 mM EDTA, 6 mM β-mercaptoethanol, 0.05 M (or 0.2 M) NaCl, 1 μg of RNA polymerase, 9 μg of T7 DNA, one dinucleotide (0.1 mM), and one NTP (0.1 mM) as indicated. After 5-min incubation at 37 °C, $(rI)_n$ was added (0.1 mg/mL) and the mixture was left for 10 min at 37 °C and then incubated for 10 min at 0 °C. RNA synthesis was then carried out for 20 min at 37 °C in the presence of 0.1 mM each of ATP, CTP, UTP, and [^{3}H]GTP (17 cpm/pmol). The reaction was terminated and worked up as described in the RNA polymerase activity assay (see Materials and Methods). The control was the standard reaction mixture without dinucleotide and NTP, and its preincubation with $(rI)_n$ was only for 10 min at 37 °C. The results are given as percentage of control.

selected promoter A_1. We have applied this method to look at the difference in promoter selection by Co- and Zn-RNA polymerase. Our results are given in Table III. The percent $(rI)_n$-resistant RNA synthesis afforded by a given combination of primer and substrate depends upon its efficiency to form a preinitiated complex at a specific promoter site. A particular feature of Co-RNA polymerase is that it preferentially selects the A_1 promoter under low salt conditions (0.05 M KCl) and the A_3 promoter under high salt conditions (0.2 M KCl). Under both conditions, the ratio of the efficiencies to initiate at $(A_1 + A_3)$ vs. A_2 is 3–4 for the cobalt enzyme as compared to about 2 for the zinc enzyme.

Further evidence that the intrinsic metal is involved in the initiation was obtained using a more specific, in vitro RNA synthesis system: the cAMP- and CRP-dependent transcription of the *lac* operon. A purified restriction fragment of *λplac5* DNA (*lac*-796), which contains the regulatory region and the proximal portion of the *z* gene of *lac* operon, was used as template (Landy et al., 1974; Gilbert et al., 1975). A five- to sixfold stimulation of total RNA synthesis as measured by incorporation of radioactive nucleotides into acid precipitable material was observed for Zn-RNA polymerase transcription in the presence of cAMP and CRP. In contrast, only a two- to threefold stimulation was obtained with Co-RNA polymerase. In the absence of cAMP and CRP, the amounts of RNA synthesized by these two enzymes were comparable.

Discussion

RNA polymerase is a key enzyme responsible for the readout of all genetic information in bacteria and, therefore, is essential for normal cell growth. *E. coli* grows normally in the zinc-depleted medium prepared by treatment with Chelex resin, indicating that the residual Zn^{2+} (10^{-8}–10^{-7} M) in the medium may still be sufficient to support the synthesis of a functionally active RNA polymerase. Addition of excess Co^{2+} (5×10^{-6} M) to the zinc-depleted medium has a temporary inhibitory effect on the bacterial growth. Recovery from the inhibition occurs, presumably, due to the ability of the cell to develop a mechanism to overcome the Co^{2+} effect. It has been reported (Leahy, 1969) that in the presence of Co^{2+}, *E. coli* K12 undergoes an extended lag period which can be reversed by addition of L-isoleucine and related amino acids. Since valine was found in this culture medium, the Co^{2+} effect was attributed to the disturbance of normal valine–isoleucine balance. More detailed studies on the synthesis of RNA and protein during inhibition of the growth of *Escherichia coli* by Co^{2+} were carried out by Blundell and Wild (1969). They found three species of RNA accumulated during inhibition, which differed slightly in sedimentation properties and ribonuclease sensitivity from normal ribosomal RNA. In this study, our results indicate that Co^{2+} can be incorporated into RNA polymerase by competing with Zn^{2+} in the medium. Thus, the Co-RNA polymerase formed could in part by responsible for the abnormal RNA synthesis in the presence of Co^{2+}.

We have used several criteria to decide that Co^{2+} actually replaced the intrinsic Zn^{2+} in RNA polymerase. (1) All enzyme preparations purified from *E. coli* grown in the presence of Co^{2+} contained a constant mole percent of Co^{2+}. No significant amount of Co^{2+} was found in RNA polymerase from cells grown in the standard medium. (2) The ratio of Co^{2+} to enzyme activity was constant across the enzyme peak when the Co^{2+}-grown enzyme was chromatographed on a DE-52 or Chelex-100 column. (3) Co^{2+}, like the tightly bound Zn^{2+}, could not be removed from the enzyme by extensive dialysis with EDTA or 1,10-phenanthroline.[2] (4) Only trace amounts of Zn^{2+} were found in Co-RNA polymerase; the total content of intrinsic metals (Zn^{2+} + Co^{2+}) is constant (2 g-atoms per mol of enzyme) in all enzyme preparations.

Quantitative analysis of Co^{2+} and Zn^{2+} in the Co-substituted enzyme preparations indicates that greater than 90% of the tightly bound Zn^{2+} in RNA polymerase has been replaced by Co^{2+}. The small population of Zn-enzyme molecules remaining is not large enough to account for the observed similarities in physical and biochemical properties between the Co- and Zn-enzymes. Furthermore, the differences we have observed would be even greater if the substitution were 100%.

The finding that the Co-substituted RNA polymerase is enzymatically as active as native enzyme is not surprising. Since the Co-substituted enzyme is produced in vivo by cells which appear to grow normally (except the initial lag), it might be expected that a vital enzyme like RNA polymerase would not be altered drastically. In fact, comparisons between the Co-substituted and native RNA polymerase have revealed that their physical and biochemical properties are very similar. Except for the properties characteristic of the metal ions, e.g. the absorption spectrum of the bound Co^{2+}, Co-RNA polymerase is almost indistinguishable from native enzyme based on such physical criteria as subunit composition, monomer–dimer equilibrium, and pH and temperature stabilities. Biochemically, both enzymes have similar specific activities on a variety of templates. Only by detailed kinetic analysis can small, yet reproducible differences in the values of apparent K_m for some specific templates be demonstrated under certain experimental conditions. This change in apparent K_m observed for DNA template by Co substitution suggests that the intrinsic metal may be involved in template binding.

Perhaps the most interesting aspect of Co-RNA polymerase concerns the initiation of RNA synthesis. Until now, the evi-

[2] From the stability constants of the metal-chelator complexes (Basolo and Pearson, 1966), it can be estimated that the association constants of the metal-enzyme complexes are at least on the order of 10^{10}–10^{12} M^{-1} for both Co- and Zn-RNA polymerase.

dence has been indirect (Scrutton et al., 1971) that the intrinsic metal of RNA polymerase plays a role in RNA chain initiation. Our finding that Co-RNA polymerase is less efficient in starting RNA chains with GTP on T7 DNA template than Zn-RNA polymerase strongly suggests that the intrinsic metal is indeed involved in the specific initiation. This is further supported by the observation that the in vitro transcription of *lac* operon by Co-RNA polymerase is less sensitive to cAMP and CRP than is the transcription by native enzyme. In the case of T7 DNA, it is known that the A_2 promoter starts with GTP while both A_1 and A_3 promoters start with ATP (Pribnow, 1975). The difference in the ratio of A starts to G starts observed with these two enzymes indicates Co-RNA polymerase cannot efficiently initiate at the A_2 promoter. This differential effect in promoter selection on T7 DNA was substantiated by measuring the relative efficiency of formation of the $(rI)_n$-resistant initiation complexes at specific promoter sites using various combinations of dinucleotides and nucleoside triphosphates (Table III).

The detailed mechanism by which the intrinsic metal in RNA polymerase effects RNA chain initiation is not known. Mechanistically, these metal ions could be involved in the binding of template or substrate, or the formation of first phosphodiester bond, or any combination of these three. That the intrinsic metal may be involved in template binding is indicated from the change in apparent K_m for T7 DNA observed by Co substitution and from the difference in promoter selection on T7 DNA between Zn- and Co-RNA polymerase described above. More direct evidence for the possible metal–template or metal–substrate interaction is provided by the absorption spectrum of Co-RNA polymerase which can be perturbed by addition of nucleoside triphosphates or a template analogue, $d(pT)_{10}$. The cobalt enzyme exhibits two major absorption bands at 584 and 703 nm, with molar absorptivity of 200 and 335 M^{-1} cm^{-1} per equivalent cobalt ion, respectively. These spectral properties suggest irregular coordinations of Co(II), presumably in nearly tetrahedral environments (Lindskog, 1970). Absorption bands near 580 nm have been recorded for Co(II)-substituted carbonic anhydrase (Lindskog, 1970), carboxypeptidase A (Coleman and Vallee, 1960), and alkaline phosphatase (Applebury and Coleman, 1969), while absorption bands near 700 nm were seen in Co(II)-substituted alcohol dehydrogenases (Curdel and Iwatsubo, 1968; Shore and Santiago, 1975). Although the actual coordination geometries require further clarification, it seems likely that the two cobalt ions in RNA polymerase may be located at heterologous sites in different environments, generating two different absorption bands. As demonstrated in Figure 3, the spectral changes induced by template and substrate are characteristically different: nucleoside triphosphates selectively perturbed the 703-nm peak, whereas $d(pT)_{10}$ altered both 703- and 584-nm peaks. These spectral changes imply that the substrate binding may involve one of the two intrinsic metal ions while the template binding may involve both. It is of interest to note that recently we have found (Wu et al., 1977) that one of the two intrinsic metal ions of RNA polymerase is located in the β' subunit which is responsible for DNA binding (Zillig et al., 1976) and that the other metal ion may be in β' or β or at the contact domain of these two subunits. The β subunit is known to contain the nucleoside triphosphate binding sites (Zillig et al., 1976; Wu and Wu, 1974). Whether the spectral changes we have observed are due to direct participation of the intrinsic metal in substrate or template binding, or whether they are due to an indirect effect such as conformational changes of the enzyme induced by substrate or template binding at sites away from the metal binding sites is not known and still remains to be investigated. To this end, the availability of an active Co-RNA polymerase, which possesses a characteristic absorption spectrum as well as paramagnetic properties, will make possible physical studies that may further define the precise role of intrinsic metal in RNA polymerase. Such studies are currently in progress in our laboratory.

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Fluorescence Energy Transfer Measurements in the Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli* with Chemically Modified Lipoic Acid†

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ABSTRACT: The lipoic acid residues of the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* have been modified with radioactive *N*-ethylmaleimide, *N*-(3-pyrene)maleimide, and *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM). The number of moles of label incorporated per 4.6×10^6 molecular weight is 47 with *N*-ethylmaleimide, 48 with *N*-(3-pyrene)maleimide, and 40 with DDPM. The last two numbers are less precise than the first because of uncertainties in the extinction coefficients of the enzyme bound labels. The overall activity of the enzyme is abolished by modification of the lipoic acid, but the enzymatic activities of pyruvate dehydrogenase and dihydrolipoyl dehydrogenase are unaltered. Direct binding measurements with 1,N^6-etheno-CoA indicate that the number of CoA specific sites of the dihydrolipoyl transacetylase is unaltered when the lipoic acid is modified with DDPM, but the dissociation constant increases about a factor of four to 218 μM (0.02 M potassium phosphate, pH 7.0, 5 °C). This suggests that lipoic acid interacts with the catalytic site of the transacetylase enzyme. Fluorescence lifetimes were used to measure fluorescence energy transfer within the enzyme complex using the following energy donors: thiochrome diphosphate, bound to the catalytic site of the pyruvate dehydrogenase enzyme; 8-anilino-1-naphthalenesulfonate, bound to the acetyl-CoA regulatory site on the pyruvate dehydrogenase enzyme; or *N*-(3-pyrene)-maleimide, bound to the lipoic acid on the dihydrolipoyl transacetylase enzyme. The energy acceptors were DDPM, bound to the lipoic acid, or FAD, bound to the dihydrolipoyl dehydrogenase enzyme. No energy transfer was observed between the modified lipoic acid groups and the labeled sites on the pyruvate dehydrogenase or the dihydrolipoyl dehydrogenase. Therefore, the distance between the lipoic acid and these sites must be greater than 40 Å assuming the emission and absorption dipoles are randomly oriented. These results and others previously reported suggest that the simple mechanism of a single lipoic acid rotating between the catalytic sites of the three enzymes is unlikely. An alternative mechanism consistent with existing data is that two or more lipoic acids are used to transfer the intermediates between the three catalytic sites of functionally coupled enzymes in a single catalytic cycle.

The purified pyruvate dehydrogenase multienzyme complex from *E. coli* has been shown to contain three component enzymes which catalyze the overall reaction (Koike et al., 1960)

$$CH_3COCO_2H + CoA + DPN^+ \rightarrow CO_2 + CH_3CO\text{-}CoA + DPNH + H^+ \quad (1)$$

The pyruvate dehydrogenase (E_1)[1] contains regulatory binding sites for acetyl-CoA and GTP plus the catalytic binding sites for pyruvate and the cofactor thiamin diphosphate. The dihydrolipoyl transacetylase (E_2) contains the binding sites for CoA and lipoic acid covalently attached through an ϵ-amino group of lysine. The dihydrolipoyl dehydrogenase (E_3) contains FAD at the active site. A model has been proposed, based on structural and chemical evidence, in which 24 polypeptide chains of E_1, 24 polypeptide chains of E_2, and 12 polypeptide chains of E_3 are arranged such that the catalytic sites for each local grouping of the three enzymes are within a sphere of radius 14 Å with the radius being defined by the lipoic acid–lysine prosthetic group. The lipoic acid group is postulated to transfer the hydroxyethyl intermediate from the active site of E_1 via a reductive acetylation of lipoic acid to the active site of E_2 where acetyl CoA is formed. The reduced lipoic acid is then reoxidized by the FAD of E_3 with the substrate DPN^+ oxidizing the FAD. A modification of this mechanism allows disulfide interchange of the intermediates between two lipoic acids which doubles the lipoic acid requirement and expands the diameter of the sphere containing the three catalytic sites to 56 Å (Koike et al., 1963).

Previous work with this multienzyme complex (Moe et al., 1974; Shepherd and Hammes, 1976; Shepherd et al., 1976; Papadakis and Hammes, 1977) has been concerned with measurements of intersubunit and intrasubunit distances between specific ligand binding sites or specific labels attached by covalent modification. The results obtained imply that the E_1, E_2, and E_3 subunits are arranged with their catalytic sites farther apart than the distances required for the single rotating lipoic acid mechanism although the intersubunit distances are

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; ϵCoA, 1,N^6-etheno-coenzyme A; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; DPN^+, nicotinamide adenine dinucleotide; DPNH, reduced DPN; E_1, pyruvate dehydrogenase; E_2, dihydrolipoyl transacetylase; E_3, dihydrolipoyl dehydrogenase; FAD, flavin adenine dinucleotide; NEM, *N*-ethylmaleimide; NPR, *N*-(3-pyrene)maleimide; $NaDodSO_4$, sodium dodecyl sulfate; Tricine, *N*-tris(hydroxymethyl)-methylglycine.