Subunit Location of the Intrinsic Divalent Metal Ions in RNA Polymerase from *Escherichia coli*†

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ABSTRACT: The DNA-dependent RNA polymerase from *Escherichia coli* possesses 2 g-atoms of tightly bound Zn ions per mol of holoenzyme ($\alpha_2\beta\beta'\sigma$). These two Zn ions are in the core enzyme ($\alpha_2\beta\beta'$), and the isolated σ subunit is essentially free of Zn. None of the individual subunits (α, β, or β') isolated from phosphocellulose chromatography of core enzyme contains significant amounts of Zn in the presence of 7 M urea, indicating that the intrinsic Zn ions have been released from core enzyme by denaturation. Similar results were also observed for the β' subunit and $\alpha_2\beta$ complex separated from urea-denatured core polymerase by Affi-Gel Blue chromatography. However, when these isolated subunits or subunit complex were dialyzed against buffer containing 10^{-5} M Zn ions to remove urea followed by dialysis against 10 mM EDTA to remove free and loosely bound Zn ions, we found that the isolated β and β' subunits contain 0.6 ± 0.3 and 1.4 ± 0.5 g-atoms of tightly bound Zn ions per mol of subunit, respectively. The isolated α subunit has less than 0.1 g-atom of Zn per mol of subunit. This finding together with the fact that the isolated subunits can be reconstituted into active enzyme strongly suggest that at least one of the two tightly bound Zn ions in RNA polymerase is located in β' subunit, while the other Zn ion may be in β', or β, or at the contact domain of these two subunits. This conclusion is further supported by studies with Co-RNA polymerase in which the two intrinsic Zn ions have been replaced by Co(II) ions. Oxidation by H_2O_2 transforms Co(II) to the exchange-inert Co(III) state and, therefore, selectively "freezes" Co ions in their respective binding sites. It was found that at least one Co(III) ion is bound tightly to the isolated β' subunit even in the presence of 6.5 M urea. The subunit locations of the two tightly bound Zn ions in RNA polymerase imply that these intrinsic metal ions may play both functional and structural roles in gene transcription.

The DNA-dependent RNA polymerase from *Escherichia coli* has been reported to be a Zn metalloenzyme (Scrutton et al., 1971). The purified RNA polymerase contains 2 g-atoms of zinc ions per mol of enzyme. These Zn ions are bound so tightly to the enzyme that they cannot be removed by dialysis against chelating reagents such as EDTA[1] or 1,10-phenanthroline. The chelating 1,10-phenanthroline, but not the nonchelating 1,7-phenanthroline, inhibits RNA chain initiation, indicating indirectly that these tightly bound Zn ions in RNA polymerase are involved in the initiation of RNA synthesis. However, we still do not know where in the enzyme these Zn ions are located and what their functions are.

To investigate the structure and function of the tightly bound metal in RNA polymerase, we have recently prepared Co-substituted RNA polymerase by growing *Escherichia coli* cells in a Co-enriched, Zn-depleted medium (Speckhard et al., 1977). The normal RNA polymerase contains two Zn and very little Co ions. On the other hand, the Co-RNA polymerase contains about two Co ions and almost no Zn. This clearly indicates that the two intrinsic Zn ions in RNA polymerase have been replaced by two divalent Co ions. The Co enzyme is catalytically as active as the Zn enzyme. The major functional difference between these two enzymes is in their ability to recognize different promoters on certain DNA templates. Another difference is that the Co enzyme exhibits a characteristic absorption spectrum in the visible region which can be perturbed by addition of substrate or template. These studies with Co-RNA polymerase further support the proposition that the intrinsic divalent metal ions in RNA polymerase play an important role in promoter recognition and specific RNA chain initiation.

This communication describes renaturation experiments that allow one to localize the intrinsic divalent metal ions in RNA polymerase with respect to the subunits of this oligomeric protein. The conclusion obtained is confirmed by an attempt to "freeze" these metal ions in their respective binding sites in native enzyme.

Materials and Methods

Chemicals. Unless otherwise stated, all biochemicals were of highest purity obtained commercially and used without further purification. Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals and ^{3}H-labeled nucleoside triphosphates from New England Nuclear. Calf-thymus DNA was the product of Worthington. Ultra Pure urea, Tris, and sodium dodecyl sulfate were obtained from Schwarz/Mann. Potassium tetrathionate was from K & K laboratories. Phosphocellulose (P 11) was from Whatman, Affi-Gel Blue (100-200 mesh) and Chelex-100 were from Bio-Rad.

Enzymes. RNA polymerase was purified from *E. coli* K12 cells (mid-log, Grand Processing) by the method of Burgess and Jendrisak (1975). The enzyme was stored in storage buffer (60% glycerol, 0.2 M KCl, 0.1 M $MgCl_2$, 0.05 M Tris-HCl (pH 8), 10^{-4} M EDTA, 10^{-3} M dithiothreitol) at −20 °C. Co-substituted RNA polymerase was purified by the same method from *E. coli* K12 cells grown in a Zn-depleted, Co(II)-enriched minimal medium as described in a recent paper (Speckhard et al., 1977), and contained >1.8 g-atoms

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[1] The abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; Affi-Gel Blue, Cibacron Blue dye covalently cross-linked to agarose; EDTA, ethylenediaminetetraacetic acid.

TABLE I: Tightly Bound Zn Ions in RNA Polymerase and Its Subunits.[a]

Preparations	Zn content (g-atoms/mol of protein)
Holoenzyme	2.1 ± 0.2
Core enzyme	2.0 ± 0.1
σ	<0.1
α	<0.1
β	0.6 ± 0.3
β'	1.4 ± 0.5 (1.5 ± 0.3)
$\alpha_2\beta$	0.7 ± 0.3 (0.6 ± 0.2)

[a] The isolation of individual subunits or subunit complexes and the determination of tightly bound zinc ions were as described in Materials and Methods. The numbers given in parentheses are Zn contents of subunits or subunit complexes isolated by Affi-Gel Blue chromatography. Zn contents of subunits separated by phosphocellulose chromatography are given without parentheses. All subunits were dialyzed against 10^{-5} M Zn ions for 2 h at room temperature and subsequently dialyzed against 10 mM EDTA to remove free and loosely bound Zn ions as described in the text. The data presented here are the average values obtained from eight different enzyme preparations.

of Co(II) ions as determined by atomic absorption spectrometry. The purity of enzyme preparations used in these studies was at least 97% as judged by dodecyl sulfate-polyacrylamide gel electrophoresis.

RNA polymerase activity was assayed by the incorporation of ^{3}H-labeled ribonucleotides into acid-insoluble material as described previously (Wu and Wu, 1973). The reaction mixture (0.25 mL) contained 0.05 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 4 mM β-mercaptoethanol, 0.4 mM each of ATP, UTP, GTP, and CTP (one labeled with ^{3}H, ~5×10^3 cpm/nmol), 30 nmol of calf-thymus DNA, and 2–5 μg of enzyme. Samples were incubated for 10 min at 37 °C, and the reactions were stopped by addition of 0.1 mL of 0.1 M sodium pyrophosphate and 5 mL of 5% trichloroacetic acid. The acid-insoluble material was collected on a Whatman GF/C glass-fiber filter, and the radioactivity incorporation was determined by liquid scintillation counting.

Isolation of Individual Subunits or Subunit Complexes. The σ subunit and core polymerase were isolated from RNA polymerase holoenzyme by the procedure of Berg et al. (1971). For isolation of subunit α, β, and β' by phosphocellulose chromatography in the presence of urea (Yarbrough and Hurwitz, 1974), core enzyme (5–10 mg) was denatured by dialysis for 2 h at 23 °C against denaturation buffer I (0.05 M Tris-HCl, pH 8, 10^{-2} M dithiothreitol, 0.1 mM EDTA, 5% glycerol, and 7 M urea). The denatured enzyme was then applied to a 1.0 × 12 cm phosphocellulose column equilibrated with denaturation buffer I containing 10^{-3} M instead of 10^{-2} M dithiothreitol. The subunits were eluted with a 300-mL linear gradient of potassium phosphate (0.01 M to 0.4 M) in the same buffer. The fractions of each subunit peak (α, β, or β') were pooled and concentrated by vacuum dialysis against reconstitution buffer or burning buffer as described later. The $\alpha_2\beta$ complex was prepared according to the method of Palm et al. (1975) from a mixture of α and β subunits (molar ratio $\alpha/\beta = 2$) isolated as described above.

For the separation of subunits by Affi-Gel Blue chromatography, a solution of 2–5 mg of core enzyme in 0.3 mL of storage buffer was mixed with ~1 mL of denaturation buffer II (0.02 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 10^{-2} M dithiothreitol, 10% glycerol, and 8 M urea), and dialyzed against the same buffer (except 0.2 mM dithiothreitol, 6.5 M urea) for 2 h at 4 °C. The mixture was then applied to a 1 × 3 cm Affi-Gel Blue column equilibrated with the dialysis buffer. The column was washed with this buffer until the eluent showed no fluorescence at 340 nm when excited at 280 nm. The protein bound to the column was then eluted with the equilibration buffer + 0.6 M KCl. The protein peak was located by measuring the fluorescence at 340 nm.

Identification of Subunits. Subunit compositions of peaks eluting from phosphocellulose of Affi-Gel Blue columns were determined by dodecyl sulfate-polyacrylamide gel electrophoresis (Shapiro et al., 1967), using RNA polymerase holoenzyme as control. When necessary, subunit identification was further confirmed by electrophoresis in a urea-containing buffer on cellulose acetate sheets as described by Palm et al. (1975).

Reconstitution of RNA Polymerase. For reconstitution, stoichiometric amounts of α, β, and β' subunits were mixed and the protein concentration was adjusted to about 0.2 mg/mL. The solutions were dialyzed against reconstitution buffer (0.05 M Tris-HCl, pH 7.9, 0.2 M KCl, 0.01 M $MgCl_2$, 1 mM EDTA, 0.02 M β-mercaptoethanol, and 20% glycerol) at 30 °C for 2–3 h before assaying for activity recovery.

Blockage and Regeneration of Sulfhydryl Groups in RNA Polymerase. To block the free SH group in RNA polymerase, the enzyme was dialyzed against 0.01 M potassium tetrathionate in N_2-flushed buffer containing 0.05 M Tris-HCl (pH 8), 0.5 M KCl, 10^{-3} M EDTA, and 20% glycerol at 23 °C for 30 min followed by dialysis overnight at 4 °C against the same buffer without potassium tetrathionate. To regenerate sulfhydryl residues, tetrathionate-treated enzyme was incubated for 1 h at 23 °C in the same buffer lacking tetrathionate but containing 0.01 M dithiothreitol.

Protein Determination. 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 Determinations. Metal content was determined using a Perkin-Elmer atomic absorption spectrophotometer Model 360, equipped with a carbon arc furnace HGA2100. Before use, all labware was acid-washed and rinsed with deionized, distilled H_2O. All buffers were made with the purest available reagents and when necessary treated with Chelex-100. Measurements were standardized by the Fisher Standard for Zn and Co which was diluted into burning buffer (0.01 M Tris-HCl, pH 8, 10 mM EDTA, 5% glycerol, 0.1 M KCl, and 0.1 mM dithiothreitol). The protein was dialyzed at least 16 h at 4 °C against burning buffer and was diluted up to 20-fold to 0.1 ~ 0.2 mg/mL with deionized, distilled H_2O before determination of its metal content. Samples, 5–10 μL, were used for Zn determination, and 10–40 μL for Co determination. Each data point consists of the average of 3–6 measurements on a given sample; the values in Table I are the averages of more than 8 data points.

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

Results

Binding of Zn Ions to Individual Subunits Isolated by Phosphocellulose Chromatography. The question of localization of intrinsic metal ions in RNA polymerase can, in principle, be answered directly by x-ray crystallographic study of the enzyme. However, for a protein with molecular weight of 500 000 like RNA polymerase, such a study is almost impossible at the present time. Therefore, we have employed an

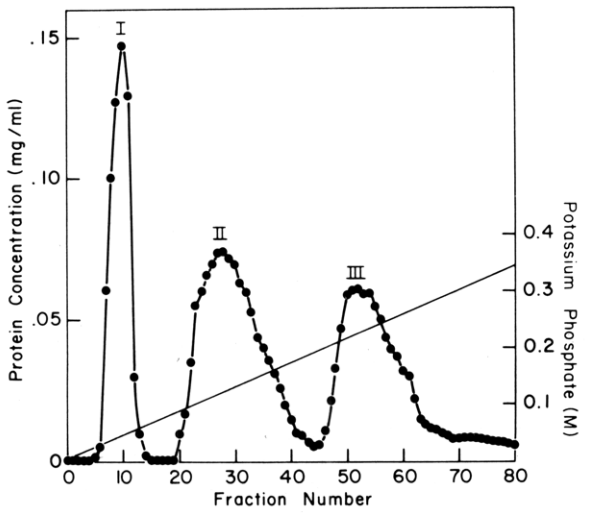

FIGURE 1: Typical elution profile of RNA polymerase subunits from a phosphocellulose column in the presence of 7 M urea.

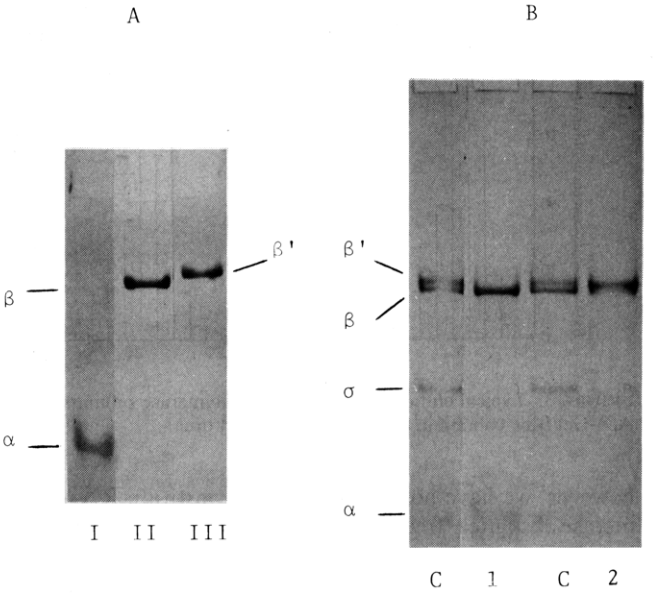

FIGURE 2: Identification of subunit composition by dodecyl sulfate–polyacrylamide gel electrophoresis. (A) Gel patterns of peaks I, II, and III obtained from the phosphocellulose chromatography shown in Figure 1. (B) Gel patterns of peaks 1 and 2 obtained from the Affi-Gel Blue chromatography shown in Figure 3. The gels marked C are holoenzyme controls.

indirect approach by asking which subunit of RNA polymerase binds zinc ions tightly. The σ subunit can be separated from the catalytically active core enzyme ($\alpha_2\beta\beta'$) by passing the RNA polymerase holoenzyme through a phosphocellulose column (Burgess, 1969). Using atomic absorption spectrometry, we observed that core polymerase contains the two tightly bound Zn ions and that isolated σ subunit is essentially free of Zn (Table I).

Now, the question is simplified to "which subunit of core polymerase contains the Zn ions?" To dissociate the individual subunits in core enzyme, one has to use denaturating reagent such as urea or guanidium hydrochloride (Yarbrough and Hurwitz, 1974; Palm et al., 1975). We have separated the individual subunits of core polymerase in the presence of 7 M urea by chromatography on phosphocellulose. Figure 1 shows the elution profile of phosphocellulose column with a phosphate gradient buffer containing 7 M urea. There are three well-separated peaks (I, II, and III). The identification of subunits in these three peaks by dodecyl sulfate–polyacrylamide gel electrophoresis is shown in Figure 2. The gel patterns of these three peaks clearly indicate that peak I is α, peak II is β, and peak III is β' subunit. We then pooled the fractions in each peak and found that none of the three peaks contained significant amounts of Zn as determined by atomic absorption. This indicates that upon denaturation by 7 M urea the tightly bound Zn ions have been released from core enzyme, and none of the individual subunits binds Zn ions in the presence of 7 M urea.

When the isolated subunits were "renatured" (removal of urea) by dialysis against reconstitution buffer, to which 10^{-5} M Zn ions was added, for 2 h at room temperature, followed by dialysis against 10 mM EDTA (in the burning buffer) over night at 4 °C to remove free and loosely bound Zn ions, we found that some of the isolated subunits did bind Zn ions tightly. Determinations of the tightly bound Zn ions in the renatured individual subunits are summarized in Table I. The isolated α subunit has less than 0.1 g-atom of Zn per mol of subunit. The isolated β subunit contains 0.3–0.9 g-atom of Zn ion, whereas the isolated β' subunit contains 0.9–1.9 g-atoms of Zn ions per mol of subunit. We have also prepared the subunit complex $\alpha_2\beta$ from the isolated α and β subunit. As expected, the $\alpha_2\beta$ complex contains 0.4–1.0 Zn ion.

Binding of Zn Ions to Individual Subunits Isolated by Affi-Gel Blue Chromatography. Halling et al. (1977) have found that one of the subunits of urea-dissociated *Bacillus subtilis* RNA polymerase core enzyme is retained on a Blue Dextran–Sepharose affinity column. When *E. coli* RNA polymerase core enzyme was denatured with 6.5 M urea and was chromatographed on an analogue of Blue Dextran–Sepharose, Affi-Gel Blue (a reactive Cibacron Blue dye covalently cross-linked to agarose), we found that the β' subunit bound to the column while the α and β subunits did not. The β' subunit was eluted by addition of 0.6 M KCl in the elution buffer. A typical elution profile is shown in Figure 3, and the dodecyl sulfate–polyacrylamide gel patterns of the two protein peaks are included in Figure 2. Indentification of β and β' subunits was confirmed by electrophoresis on cellulose acetate as described by Palm et al. (1975). Atomic absorption analysis revealed that little Zn ions were associated with the proteins in these two peaks in the presence of 6.5 M urea. After removal of urea by dialysis against reconstitution buffer to which 10^{-5} M Zn ions had been added followed by dialysis against 10 mM EDTA (in burning buffer), peak 2 (β' subunit) showed the presence of 1.2–1.8 Zn ions per subunit while peak 1 (α and β subunits) had 0.4–0.8 Zn ion per $\alpha_2\beta$ subunit complex. These values, which are also given in Table I, are consistent with those observed for the individual subunits isolated by phosphocellulose chromatography described above.

Reconstitution of Active Enzyme by Isolated Subunits. The renatured individual subunits or subunit mixtures isolated by phosphocellulose or Affi-Gel Blue chromatography can be reconstituted into active core enzyme by incubating equivalent amount of subunits (2α, 1β, $1\beta'$) for 2–3 h at room temperature in reconstitution buffer (without adding Zn). The specific activity of the reconstituted enzyme ranged from 30 to 50% of that of native core enzyme using calf-thymus DNA as template. Enzymes of similar specific activity could also be reconstituted by co-dialysis of equivalent amounts of the denatured individual subunits (in urea) against reconstitution buffer at room temperature for 2–3 h. Addition of 10^{-5} M Zn ions in the reconstitution buffer did not significantly alter the specific activity. Even without the deliberate addition of zinc,

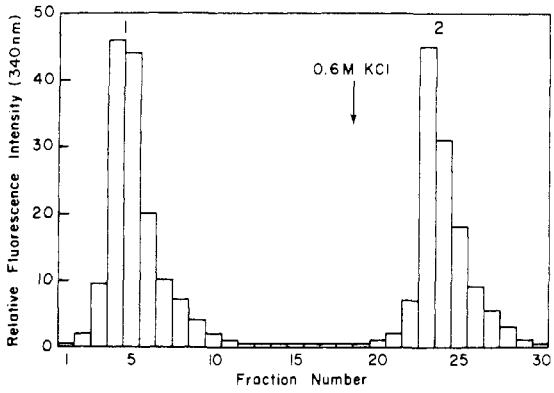

FIGURE 3: Typical elution profile of RNA polymerase subunits from a Affi-Gel Blue column in the presence of 6.5 M urea.

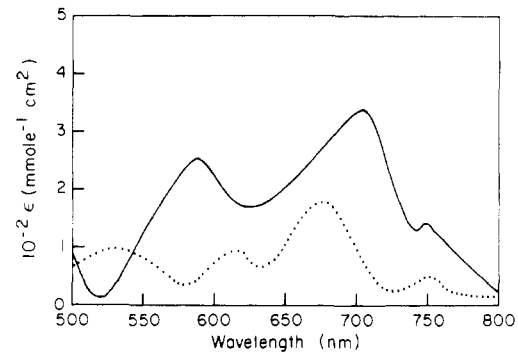

FIGURE 4: Visible absorption spectra of Co-RNA polymerase in the presence and absence of hydrogen peroxide. The sample was 10 mg/mL of Co-RNA polymerase in 0.05 M Tris-HCl (pH 7.9) buffer containing 0.15 M KCl, 0.01 M $MgCl_2$, 0.1 mM dithiothreitol, and 0.1 mM EDTA. The reference was the same concentration of Zn-RNA polymerase in the same buffer. (—) No H_2O_2; (···) after H_2O_2 treatment as described in the text.

however, we have not been able to reconstitute RNA polymerase absolutely free of Zn ions, since there were enough Zn ions in the reaction mixtures and buffers to repopulate the apoenzyme with Zn. (Both the reconstitution buffer and the assay mixture contain high concentrations of sulfhydryl reagents which are strong chelators of Zn ions.) In addition, we have noted that the ability of renatured individual subunits to bind Zn ions decreased upon aging or elimination of thiol reagents which were normally present in the subunit preparations to prevent oxidation of the enzyme during aging. This may be the reason for the relatively large variations of the Zn content of the isolated subunits shown in Table I. The aged or oxidized subunit preparations also had a decreased ability to be reconstituted into active enzyme. The data on Zn binding to isolated individual subunits in Table I and the fact that these subunits can be reconstituted into active enzyme strongly suggest that at least one of the two tightly bound Zn ions in *E. coli* RNA polymerase is located in β' subunit. The other Zn ion may be in β', or β, or at the contact domain of these two subunits. Nevertheless, this conclusion is not completely beyond doubt since the isolated subunits may assume a conformation different from that which they have in native enzyme and thus may have different metal binding properties. To resolve this uncertainty, we have attempted to "freeze" the intrinsic divalent metal ions in their binding sites in native enzyme using the Co-substituted RNA polymerase.

Oxidation of Co(II) in RNA Polymerase to Co(III) by H_2O_2. It has been shown that the Co(II) ions in Co-substituted alkaline phosphatase (Anderson and Vallee, 1975), carbonic anhydrase (Shinar and Navon, 1974), and carboxypeptidase A (Kang et al., 1972) can be oxidized by treatment with H_2O_2. The oxidation transforms Co(II) from the exchange-labile (d^7) to the exchange-inert (d^6) Co(III) state and, therefore, offers a method to "freeze" cobalt ions in their respective binding sites. The assumption was made that the oxidation does not change the location of cobalt on the enzyme. When Zn- or Co(II)-RNA polymerase ($\sim 10^{-5}$ M) in storage buffer was incubated with a 20-fold molar excess of H_2O_2 at room temperature for 30 min, the enzymatic activity was lost completely. However, if Zn-RNA polymerase was pretreated with potassium tetrathionate to block free sulfhydryl groups (Yarbrough and Wu, 1974) and then reacted with H_2O_2, the enzymatic activity could be completely restored by addition of excess β-mercaptoethanol. This implies that the inactivation of Zn-RNA polymerase was due to the oxidation of free sulfhydryl groups in the enzyme and that other functionally important amino acid residues were not significantly altered by treatment with H_2O_2. No restoration of enzymatic activity by β-mercaptoethanol was observed for the SH-blocked Co(II)-RNA polymerase after reaction with H_2O_2. More direct evidence[2] that it was the Co(II) ions in the enzyme which were oxidized is provided by the distinct change in absorption spectrum of Co-RNA polymerase in the visible region from 500 to 800 nm (Figure 4). Both the 586- and 703-nm peaks of Co(II)-RNA polymerase vanished upon treatment with H_2O_2 and were replaced by a major peak at 675 nm ($\epsilon \simeq 190$ per Co ion) and three minor peaks. Reaction of Co(II)-RNA polymerase with H_2O_2 did not result in significant loss of the tightly bound Co ions (Table II). Furthermore, when the oxidized enzyme was denatured and the subunit mixture was subjected to Affi-Gel Blue chromatography, one Co(III) ion was still bound to the β' subunit even in the presence of 6.5 M urea. This observation further supports our contention that at least one of the intrinsic divalent metal ions is located in the β' subunit of native RNA polymerase.

TABLE II: Cobalt Content of Co-RNA Polymerase and Its Subunits in the Presence and Absence of H_2O_2.[a]

	$-H_2O_2$	$+H_2O_2$
Holoenzyme	1.9 ± 0.2	1.8 ± 0.3
Core enzyme	1.7 ± 0.3	1.7 ± 0.2
β'	<0.1	1.4 ± 0.2
$\alpha_2\beta$	<0.1	0.2 ± 0.1

[a] The determination of Co contents of holo- and core enzyme by atomic absorption analysis was as described in Materials and Methods. The Co contents of β' subunit and $\alpha_2\beta$ complex were measured immediately after separation by Affi-Gel Blue chromatography in the presence of 6.5 M urea.

Discussion

Zn has been shown to be associated with many nucleotidyltransferases including terminal deoxynucleotidyltransferase, and DNA and RNA polymerases from both prokaryotic and eukaryotic cells (Chang and Bollum, 1970; Slater et al., 1971; Scrutton et al., 1971; Valenzuela et al., 1973; Auld et al., 1974; Coleman, 1974). Its precise function remains unknown, however. Studies with terminal nucleotidyltransferase (Chang and Bollum, 1970) and DNA polymerase I (Springgate et al., 1973) indicate that the enzyme-bound Zn

[2] A more definite demonstration of the formation of Co(III) species is provided by our preliminary experiment (Speckhard and Wu, unpublished result) showing that the EPR signal of Co(II)-RNA polymerase diminished after the enzyme was treated with H_2O_2.

ion interacts with DNA, possibly by coordination with the 3′-OH group of the primer. It has been proposed that the Zn ion may participate directly in catalysis by acting as a Lewis acid to facilitate the deprotonation of the 3′-OH, a necessary step for nucleophilic attack on the α-phosphorus of the incoming deoxynucleoside triphosphate. Although RNA polymerase does not require a primer for initiation of RNA synthesis, it has been suggested (Mildvan, 1974) that the Zn ion may position itself at the 3′-OH group of the terminal nucleotide in the growing RNA chain and thereby play a catalytic role similar to that proposed for the other nucleotidyltransferases. In addition to the possible catalytic role, the Zn ions in RNA polymerase may also be involved in recognition of certain specific promoter sites on DNA template. By substituting the intrinsic Zn ions with Co(II) ions, we have found in a previous study (Speckhard et al., 1977) that the enzyme utilizes the A_2 promoter of T7 DNA less efficiently than the $A_1 + A_3$ promoters. It was also observed that the in vitro transcription of the *lac* operon by Co-RNA polymerase is less sensitive to cAMP and cAMP receptor protein than is the transcription by Zn-RNA polymerase. The physical basis of these observations could be a stabilization of the DNA-enzyme complex or an alteration of the conformation of the DNA template mediated through the metal ions. In this connection, it is of interest to note that Zn ion has been shown to assist the reversible unwinding and rewinding of the double helical DNA induced by heating and cooling (Shin and Eichhorn, 1968). Another possible role for the intrinsic Zn ions is the maintenance of the proper subunit arrangement. Thus Zn ion could affect the function of the enzyme indirectly.

The present studies on the subunit locations of the two intrinsic Zn ions in *E. coli* RNA polymerase suggest that these metal ions may serve some or all of the three roles discussed above. Our results clearly indicate that at least one of the two Zn ions is located in the β' subunit. Several lines of evidence indicate that β' is the subunit responsible for binding DNA template. The isolated β' subunit is able to bind DNA (Sethi and Zillig, 1970; Fukuda and Ishihama, 1974; Yarbrough and Hurwitz, 1974), while the DNA binding capacity of other subunits is insignificant. Furthermore, two mutants of RNA polymerase with temperature-sensitive mutation in β' have been demonstrated to be either decreased in DNA binding ability (Panny et al., 1975) or unable to melt out promoter sites (Gross et al., 1976). If the intrinsic Zn ion in β' subunit is indeed involved in template binding or promoter selection, it will be of interest to examine whether the Zn ion is still present in the mutant enzyme. The location of the second Zn ion is less clear, owing to the relatively wide variation in Zn content of isolated subunits shown in Table I. In spite of the uncertainty, we may conclude that the second Zn ion is either located in β' or β subunit. The evidence in favor of its location in β subunit comes from our observation that addition of nucleoside triphosphate perturbs the visible absorption spectrum of Co-RNA polymerase (Speckhard et al., 1977) since the β subunit is known to contain the nucleotide triphosphate binding sites as shown by affinity labeling experiments (Nixon et al., 1972; Frischauf and Scheit, 1973; Wu and Wu, 1974). In addition, 1,10-phenanthroline appeared to compete with GTP for binding to the initiation sites on core enzyme (Scrutton et al., 1971), suggesting that this nucleotide binding site (in β subunit) may contain tightly bound Zn. An alternative interpretation of the data in Table I is that the second Zn ion is close to the subunit contacts between β and β', which may lead to partial binding of the metal ion by isolated β or β' subunit. This possibility could imply a structural role of the Zn ion in maintaining proper quaternary structure for transcription, and is particularly interesting for an allosteric enzyme like RNA polymerase in which subunit contacts are necessary for the transfer of conformational information.

The *Bacillus subtilis* RNA polymerase has a subunit composition similar to that of the *E. coli* enzyme (Avila et al., 1971; Shorenstein and Losick, 1973). Recently, Halling et al. (1977) reported that the *B. subtilis* RNA polymerase also contains two atoms of tightly bound Zn per enzyme molecule. When the urea-dissociated subunits of the *B. subtilis* enzyme were passed through a Blue Dextran-Sepharose column, they found that one subunit, designated as β because of its molecular weight, was retained on the column. In addition, the two Zn ions were associated with the β subunit. An experimental observation of Halling et al. which appears to be different from ours is that the denaturation of core enzyme by urea did not result in loss of the tightly bound Zn ions. This discrepancy might be attributed to the difference in the subunit structure between these two enzymes and hence their modes of Zn binding may be different. It should be noted, however, that Halling and Doi (unpublished observation cited in Halling et al. (1977)) also observed that incubation of the *B. subtilis* RNA polymerase for several days in 7 M urea resulted in complete release of the Zn ions. The finding that Zn ions are present in the β subunit prompted Halling et al. (1977) to conclude that it is consistent with the evidence that this subunit is directly involved in the phosphodiester bond formation. It was discovered later, however, that the largest subunit in the *B. subtilis* RNA polymerase (designated as β' by molecular weight) determines the rifampicin-resistant phenotype (see Note Added in Proof in Halling et al., 1977). Thus this subunit should be called the β subunit and the Zn-containing subunit should be called the β' subunit according to the subunit nomenclature given to the *E. coli* RNA polymerase. Therefore, the subunit location of Zn ions in the *B. subtilis* enzyme reported by Halling et al. is consistent with that of the *E. coli* RNA polymerase described here.

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Effect of Aminoacyl Transfer RNA Synthetases on H-5 Exchange of Specific Pyrimidines in Transfer RNAs†

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ABSTRACT: The effect of bound aminoacyl-tRNA synthetases on tritium incorporation into the C-5 positions of pyrimidines in transfer RNAs was studied at 37 °C. In free *E. coli* $tRNA^{Ile}$ and $tRNA^{Tyr}_2$, labeling rates of the mixture of total uridines and of total cytidines are comparable to those of the corresponding free mononucleosides. Also, for both tRNAs labeling of the composite of cytidines is unaffected by bound cognate synthetase. In contrast, labeling rates of the uridines are accelerated in the presence of cognate enzyme about 3-fold in each case. On the other hand, when $tRNA^{Tyr}_2$ is incubated with the noncognate Ile-tRNA synthetase, there is no accelerated uridine labeling; this shows that the accelerated labeling is due to specific synthetase–tRNA interactions. Further investigation of the $tRNA^{Ile}$ and $tRNA^{Tyr}_2$ systems was carried out to determine if the enhancement in overall uridine labeling is due to effects spread over many specific sites, or to enhanced labeling concentrated at only a few loci. It was found that in both instances almost all specific uridines in the respective sequences are unaffected by bound cognate enzyme. On the other hand, pronounced enhanced (10-fold or more) exchange is observed at U8 in $tRNA^{Ile}$ and U8 (and, additionally or alternatively, U9) in $tRNA^{Tyr}_2$. This raises the possibility of a special interaction, such as a transient covalent bond, between bound synthetases and tRNAs at U8. Since a uridine or thiouridine occurs at position 8 in all tRNAs sequenced to date, the results suggest a role for this residue in most or all synthetase–tRNA complexes. Preliminary results on other systems are compatible with this possibility. In the three-dimensional tRNA structure this residue occurs at a position previously proposed as within the area of enzyme–tRNA contacts.

In an earlier set of investigations we used tritium as an atomic size probe to explore transfer RNA structure in solution as well as structural features of a complex of a specific tRNA with its cognate aminoacyl tRNA synthetase (Gamble and Schimmel, 1974; Gamble et al., 1976; Schoemaker et al., 1976; Schoemaker and Schimmel, 1976). These studies took advantage of the slow exchange of the hydrogen at the C-8 position in purine nucleotides. Transfer RNA was exposed to tritiated water until sufficient tritium was incorporated into its various purine nucleotide units. Subsequently the tRNA was purified away from free and loosely bound tritium, digested with specific nucleases, and chromatographed so as to obtain purine units from known sections in the sequence. By determination of the specific activities of these purines, it was possible to measure labeling rates of purine nucleotides distributed throughout the structure. These studies showed that the labeling rates are extremely sensitive to secondary and tertiary structure, with the labeling rate of an A or G depending markedly on its location in the sequence. Moreover, when $tRNA^{Ile}$ is incubated with Ile-tRNA synthetase, tritium labeling of specific purines in the sequence is blocked, or retarded, by the bound enzyme. The enzyme–tRNA contact points revealed by the tritium labeling approach (Schoemaker and Schimmel, 1976) har-

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