Fluorescence Resonance Energy Transfer Studies on the Proximity Relationship between the Intrinsic Metal Ion and Substrate Binding Sites of Escherichia coli RNA Polymerase*

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DNA-dependent RNA polymerase from Escherichia coli contains 2 mol of zinc/mol of holoenzyme (αββ′α) with one zinc each in the β and β′ subunits. A new method to substitute selectively the zinc in the β subunit was developed by the inactivation of RNA polymerase with 0.25 M NaNO₂, 1 M NaCl, 1 mM dianinocyclohexane-tetraacetic acid, and 0.1 M dithiothreitol followed by reconstitution with Co(II), Cd(II), or Cu(II). The hybrid Co-Zn, Cd-Zn, or Cu-Zn RNA polymerase thus obtained retains, respectively, 91, 88, and 50% enzyme activity of the reconstituted Zn-Zn RNA polymerase. Co-Zn RNA polymerase exhibits absorption maxima at 395 and 465 nm, and Cu-Zn RNA polymerase at 637 nm (ε = 815 M⁻¹ cm⁻¹).

1-Aminonaphthalene-5-sulfonic acid (AmNS) derivatives of ATP, UTP, and dinitoside monophosphates (dNMPs), UpA or ApU, were synthesized with AmNS attached to NTP via a γ-phosphoamidate bond or to dNMPs via a 5'-secondary amine linkage. Since the fluorescence emission maxima of (5'-AmNS)UpA, (γ-AmNS)ATP, and (γ-AmNS)UTP at 445, 464, and 464 nm, respectively, when excited at 340 nm, overlap the 465-nm absorption band of Co-Zn RNA polymerase, the spatial relationship between fluorescence substrate analogs and the intrinsic Co(II) in Co-Zn RNA polymerase was studied by fluorescence resonance energy transfer technique. The fluorescence of the initiator, (5'-AmNS)UpA, and elongator, (γ-AmNS)UTP, of the RNA chain, was quenched 20.3 and 7.1%, respectively, by the addition of saturation concentration of Zn-Zn RNA polymerase, and 21.3 and 14.7%, respectively, by the addition of template, poly(dA-dT). The fluorescence of (5'-AmNS)UpA and (γ-AmNS)UTP was quenched 81.8 and 80.6%, respectively, by the addition of the saturation concentration of Co-Zn RNA polymerase in the absence of template, and 82.7 and 82.9% in the presence of template. On the basis of respective R₀ values of 21.3 and 21.9 Å for the (5'-AmNS)UpA-Co and (γ-AmNS)UTP-Co pairs, the distances from Co(II) to the initiation site and to the elongation site were calculated to be 17.4 and 17.5 Å, respectively, in the absence and 17.2 and 17.4 Å in the presence of template.

RNA polymerase from Escherichia coli is a multisubunit enzyme (αββ′α) whose structure, function, and regulation are the focus of many extensive studies (1-6). The enzyme contains two zinc ions/enzyme molecule (7), the substrate binding subunit, β, and template binding subunit, β′, each containing one (8, 9). In addition, this enzyme possesses two substrate binding sites, the initiation and the elongation site (10, 11). The initiation site binds the initiator, mainly a purine nucleotide (12) or dinucleoside monophosphate with the 5'-terminal base being either adenine or guanine (13). Wilkinson et al. (14, 15) have shown that dinucleoside monophosphate can fulfill the requirement for an initiation factor under certain conditions when directed by a specific DNA template. On the other hand, the elongation or polymerization site can bind any of the four nucleoside triphosphate substrates (12), with a specificity determined by the DNA template. It has been shown that more information on these catalytic sites can be obtained by examining the structure and conformation of bound nucleotides, both at the initiation site, using the dinucleoside monophosphate initiator, and at the elongation site, using mononucleoside triphosphates in the presence of specific DNA template (16, 17).

Our earlier nuclear magnetic resonance (NMR) studies on Co(β)-Zn(β′) RNA polymerase¹ obtained by in vitro metal substitution (18) indicated that the Co(II) ion is located at the initiation site of the enzyme and is in direct coordination (<4 Å) with the base moiety of the nucleotide substrate in the absence (18) and presence (19) of DNA. These results suggest that the metal ion in the β subunit plays a regulatory role in the recognition and orientation of the initiation nucleotide into a stereospecific position for catalysis.

¹ The abbreviations used are: Co-Zn, Cd-Zn or Cu-Zn RNA polymerases, RNA polymerases containing one intrinsic cobalt, cadmium, or copper ion, respectively, and one intrinsic Zn-Zn RNA polymerase, RNA polymerase containing two intrinsic zinc ions; AmNS, 1-aminonaphthalene-5-sulfonic acid; (γ-AmNS)ATP, adenosine-5'-triphosphory-γ-1-(5-sulfonic acid)naphthylamidate; (γ-AmNS)UTP, uridine-5'-triphosphory-γ-1-(5-sulfonic acid)naphthylamidate; NTP, nucleoside triphosphates; UpA, uridylyl(3',5')adenosine; (5'-AmNS)UpA, 5'-(1-(5-sulfonic acid)naphthylamino)uridylyl(3',5')adenosine; pol[y(dA-dT)], double-stranded alternating co-polymer of deoxyadenylate-deoxythymidylate; DTT, dithiothreitol; DCTA, trans-1,2-diaminocyclohexane-N,N′,N″,N‴tetraacetic acid; FRET, fluorescence resonance energy transfer; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; CD, circular dichroism.

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In our previous communications (20, 21), we reported the preparation and spectroscopic properties of dinucleoside monophosphates, e.g. (5'-AmNS)UpA/ApU, and mononucleoside triphosphate, e.g. (γ-AmNS)ATP/UTP, with a fluorophore, 1-amino-(5-sulfonic acid) (AmNS), labeled at the 5'-terminus and γ-phosphorus, respectively. These fluorescent substrate analogs have their emission maxima around 445 and 464 nm, respectively, when excited at 340 nm. These emission spectra overlap with the absorption spectrum of Co-Zn RNA polymerase (18). Moreover, these analogs can be incorporated into RNA product by E. coli RNA polymerase in the presence of poly(dA-dT) template (24). The Km (μM) and Vmax (nmol/min/mg of enzyme) values are 5 and 17 for (5'-AmNS)UpA versus 2.5 and 25 for UpA (21), and 82 and 42 for (γ-AmNS)UTP versus 53 and 72 for UTP (21, 22). These facts enable them to be used as substrate initiator (analogs of dinucleoside monophosphate) and elongator (analogs of mononucleoside triphosphate) in the study of transcription by fluorescence spectroscopy.

In this study, we report a new procedure to selectively substitute the metal ions in the β subunit of E. coli RNA polymerase with other metal ions (cobalt, cadmium, or copper) under noncondensing conditions yielding metal hybrid Co-Zn, Cd-Zn, or Cu-Zn RNA polymerase with 91, 88, and 50% enzyme activity, respectively. Our studies focus on the interactions between the Co-Zn RNA polymerase and various fluorescent derivatives of dinucleotide and mononucleotide in the absence and presence of the template during RNA synthesis. We have studied two reaction steps: the initiation step, involving the interaction of Co-Zn RNA polymerase and (5'-AmNS)UpA, and the elongation step, involving Co-Zn RNA polymerase and (γ-AmNS)UTP by absorption and fluorescence spectroscopy. The Co-Zn RNA polymerase was used as an energy acceptor, while the fluorescent dinucleoside monophosphate or mononucleoside triphosphate was employed as an energy donor in the fluorescence resonance energy transfer (FRET) studies. The spatial relationship between the cobalt in Co(β)-Zn(β') RNA polymerase and the initiator, (5'-AmNS)UpA, and that between the cobalt to the elongator, (γ-AmNS)UTP, are delineated.

**EXPERIMENTAL PROCEDURES**

**Materials—Nucleoside triphosphates (NTPs) and uridylyl-(3',5')adenosine monophosphate (UpA) were obtained from Pharmacia Biotechnology, Inc. and [14]UTP from ICN Pharmaceuticals. Ultrapure urea, Tris, and AmNS were purchased from Schwarz/Mann, Calif, thymus DNA (type I, highly polymerized), DCTA, EDTA, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were products of Sigma. Affi-Gel blue (100-200 mesh) and Cheloz 100 were obtained from Bio-Rad. All metal salts were purchased from J. T. Baker Chemical Co., and the standard metal solutions for atomic absorption analysis were from Fisher. All other biochemicals and chemicals were of the highest purity obtainable commercially.

**Purification of RNA Polymerase and Its Subunits—RNA polymerase was purified by the method of Burgus and Jendrisak (23) from E. coli MRE-600 cells (Grain Processing Co., Muscatine, IL). The enzyme was at least 98% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was stored in 50 mM Tris-Cl (pH 7.8), 50% glycerol, 0.2 M KCl, 10 mM MgCl₂, 0.1 mM EDTA, and 0.1 mM DTT at 20 °C. The protein concentration was determined by both absorption spectroscopy using an extinction coefficient (ε₁₀₀₀) of 8.0 (24) and by the Bradford (25) dye binding technique. The preparation of core RNA polymerase into αβ subcomplex and β' subunit was performed by Affi-Gel blue column chromatography as described earlier (8). Peak I (αβ) was eluted by buffer A (29 mM Tris-Cl (pH 8.0), 10 mM MgCl₂, 0.2 mM DTT, 10% glycerol, and 7 M urea) and peak II (β') by buffer A plus 0.6 M KCl.

**Biochemical Assays—** The enzymatic activities of RNA polymerase and the metal-substituted RNA polymerases were assayed by the incorporation of H-labeled ribonucleoside monophosphate into acid-insoluble material using calf thymus DNA as a template (26). The enzyme assay mixture (0.25 ml) for studying the utilization of mononucleotide triphosphates and their fluorescent analogs contains 5 μM of RNA polymerase, 50 mM Tris-Cl (pH 7.9), 10 mM MgCl₂, 0.1 mM β-mercaptoethanol, 0.4 mM of each CTP, GTP, [3H]UTP (3725 cpm/nmol) (or γ-AmNS)-[3H]UTP and ATP (or γ-AmNS)-ATP, and 2 mM poly(dA-dT) template and fluorescent derivatives of dinucleotide and mononucleotide in the absence and presence of the template during RNA synthesis. We have studied two reaction steps: the initiation step, involving the interaction of Co-Zn RNA polymerase and the initiator, (5'-AmNS)UpA, and the elongation step, involving Co-Zn RNA polymerase and (γ-AmNS)UTP by absorption and fluorescence spectroscopy. The Co-Zn RNA polymerase was used as an energy acceptor, while the fluorescent dinucleoside monophosphate or mononucleoside triphosphate was employed as an energy donor in the fluorescence resonance energy transfer (FRET) studies. The spatial relationship between the cobalt in Co(β)-Zn(β') RNA polymerase and the initiator, (5'-AmNS)UpA, and that between the cobalt to the elongator, (γ-AmNS)UTP, are delineated.
cells (0.3 × 0.3 cm, 120-μl capacity) were used for all the experiments. Samples were prepared and incubated for 10 min at 37 °C before their spectra were recorded. The fluorescence records were the average of at least five experiments. All spectrophotometric measurements were carried out at 37 °C in a temperature-controlled cuvette holder.

Quantum Yield (Qd) of Samples—Corrected emission spectra for the reference standard and sample were recorded. The Qd of the sample was calculated according to the following equation (30-33).

\[ Q_d = Q_0 \frac{F_d \Delta \lambda \times q_d \times \lambda_d}{F_r \Delta \lambda \times q_r \times \lambda_d} \]  

(1)

The subscripts s and d refer to standard and unknown, respectively; \( F \) is the relative fluorescence intensity and \( \Delta \lambda \) is the wavelength interval over which the integration was carried out (in this case, \( \Delta \lambda = 1 \) nm); \( q \) equals relative photon output of the source at the excitation wavelength, taken directly from the calibration curve once the slits were kept constant. \( A \) is the absorbance at the excitation wavelength. Solutions with the absorbance at either excitation or emission wavelength <0.01 were used for measurement to reduce the inner filter effect. The corrected emission spectra (\( Q_d = 280 \) nm) for Zn-Zn RNA polymerase, Co-Zn RNA polymerase, and N-acetyl \( \gamma \)-tryptophanamide (\( Q_d = 0.28 (34) \)) were recorded. The \( Q_d \) values for Zn-Zn and Co-Zn RNA polymerases in a buffer of 50 mM Tris-Cl (pH 7.9), 10 mM MgCl₂, and 0.1 mM \( \beta \)-mercaptoethanol were then determined to be 0.172 and 0.233, respectively. The respective reported absorbance at 460 nm) using quinine sulfate in 0.1 M H₂SO₄ as standard (\( Q_d = 0.55 \)) (36).

In the Förster theory of dipole-dipole energy transfer (37), the transfer efficiency \( (E) \) is related to the distance, \( r \) (Å), between the donor and the acceptor by

\[ r = R_0 \left( \frac{1}{E} - 1 \right)^{1/6} \]  

(2)

or

\[ E = \frac{r^6}{r^6 + R_0^6} \]  

(3)

where \( F_d \) and \( F_r \) are the fluorescence intensities of the donor in the presence of Zn-Zn RNA polymerase and Co-Zn RNA polymerase, respectively.

\( R_0 \) the distance (Å) at which the transfer efficiency equals 50%, is given by the following equation,

\[ R_0 = 9.79 \times 10^3 ((J)(Q_d)(r^6)(\epsilon^2))^{1/6} \]  

(4)

where \( \epsilon \) is the refractive index of the medium and \( r^2 \) is the orientation factor. The uncertainty in the estimation of the orientation factor \((\epsilon^2 \approx 2/3)\) was assumed to be negligible as in the case of Co(II) being used as an energy acceptor (37, 38). The spectral overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor was approximated by the following summation.

\[ J(M^{-1} cm^2) = \frac{\int F_d(\lambda) \cdot \epsilon_d(\lambda) \cdot \lambda^4 \cdot \Delta \lambda}{\int F_d(\lambda) \cdot \Delta \lambda} \]  

(5)

where \( F_d(\lambda) \) and \( \epsilon_d(\lambda) \) are the relative fluorescence intensity (%) of the donor and the molar extinction coefficient \( (M^{-1} cm^2) \) of the acceptor, respectively, and \( \lambda \) is the wavelength in nm. The summation was taken at each nm interval, \( \Delta \lambda \). We have designed a computer program to calculate \( Q_d \), \( E \), \( R_0 \), \( J \), and \( r \) values (see "Appendix").

The lifetime, \( r_{A} \) (ns), of the donor-acceptor complex can be calculated by

\[ r_{A} = r_{A} (1 - E) \]  

(6)

where \( r_{A} \) is the lifetime for the donor in the absence of acceptor.

The rate constant of energy transfer, \( k_T \), between donor and acceptor can be calculated as follows

\[ k_T (s^{-1}) = \frac{(1/r_{A})E}{1 - E} \]  

(7)

RESULTS

Absorption Spectra of Fluorescent Nucleotides—The absorption spectra of (5′-AmNS)UpA, (γ-AmNS)UTP, and (γ-AmNS)ATP are shown in Fig. 1. They display a broad band near 330–340 nm with an isosbestic point at 314 nm which was associated with the naphthalene ring, a maximum around 210–244 nm, and a distinct shoulder at 265–275 nm due to the base rings. The AmNS moiety in ATP and UTP is attached via a \( \gamma \)-phosphoamidate bond and that in UpA via a 5′-secondary amine linkage.

Metal Hybrid RNA Polymerases—Intrinsic metal ions in E. coli RNA polymerase were removed in a dissociation buffer which contains Tris succinate (pH 6), 1 mM NaCl, 1 mM DCTA, 0.25 mM NaNO₂, and 0.1 mM DTT. In this method, nitrate was used as a passivator (30, 39) and DCTA as a strong chelator for the transition metal ions. The enzyme was inactive after 24-h dialysis against the dissociation buffer. The aepozyme was then reconstituted by dialysis at 4 °C against reconstitution buffer in the presence of 2 mM sulfate salts of Co(II), Cd(II), and Cu(II), respectively, yielding Co-Zn, Cd-Zn, and Cu-Zn RNA polymerase. The respective enzyme activities of Co-Zn, Cd-Zn, and Cu-Zn RNA polymerases were 91, 88, and 50% that of the reconstituted Zn-Zn RNA polymerase enzyme. To examine whether the NO₃⁻ generated in situ from the reduction of NaNO₂ by sulfhydryl group or the NaNO₂ added exogenously inactivates RNA polymerase, we have compared the enzyme activity of the Zn-Zn RNA polymerase with 0.25 mM NaNO₂ or NaNO₃ included in the dissociation buffer without DTT. After 24-h dialysis against dissociation buffer without DTT, the RNA polymerase activity was found to be 63 and 95% in the presence of NaNO₂ and NaNO₃, respectively. These results indicated that NaNO₃, but not NaNO₂, reacted with the sulfhydryl groups of RNA polymerase yielding partially inactive enzyme which may subsequently release the zinc ions with the aid of DCTA.

The cobalt, cadmium, and copper content of the metal-hybrid enzymes obtained above was determined to be 1 mol of metal/mol of enzyme. The subunit location of the metal which was being substituted in the reconstituted RNA polymerases was determined by chromatography of the urea-treated core (αββ') RNA polymerase on an Affi-Gel blue column as described earlier (8) and by atomic absorption analyses of the metal content in αββ' subcomplex and β' subunit. The results showed that the substituted metal was incorporated into the

FIG. 1. Absorption spectra of (γ-AmNS)UTP (a), (γ-AmNS)ATP (b), and (5′-AmNS)UpA (c). The inset shows the complete spectrum of (5′-AmNS)UpA. The compounds were in 10 mM Tris-Cl (pH 8) buffer.
β subunit of RNA polymerase since α subunit does not contain metal (8).

**Absorption Spectra of Metal Hybrid RNA Polymerases**—The absorption spectra of Cu-Zn and Co-Zn RNA polymerase are shown in Figs. 2 and 3, respectively. The spectrum of Co-Zn RNA polymerase exhibits two peaks at 395 and 465 nm as that observed earlier for the Co-Zn RNA polymerase obtained by the urea denaturation method (16). The spectrum of Cu-Zn RNA polymerase first recorded here shows a broad band around 637 nm ($\varepsilon = 815 \text{ M}^{-1} \text{ cm}^{-1}$).

**Use of Fluorescent Nucleotides as Substrates for RNA Synthesis**—The fluorescent analogs of UTP and ATP were studied for their ability to support the DNA-dependent RNA synthesis by E. coli RNA polymerase using calf thymus DNA as a template. The incorporation of (γ-AmNS)UTP and (γ-AmNS)ATP into RNA product in 10 min at 37°C under the conditions for standard enzyme activity assay as stated under “Experimental Procedures” was found to be 73 and 60%, respectively, of that with the corresponding unmodified nucleotides. These results are in good agreement with those reported earlier (22). Furthermore, we have determined the $K_d$ values of (γ-AmNS)UTP and (5'-AmNS)UpA for both Zn-Zn and Co-Zn RNA polymerases by fluorescence titration as shown in Fig. 4 for (5'-AmNS)UpA (0.1 μM) with varying concentration of Zn-Zn (A—A) and Co-Zn (A—O) RNA polymerase (RPase) in 80 mM Tris-Cl (pH 8), 10 mM MgCl₂, 50 mM KCl, and 2 mM DTT. The excitation and emission wavelength is 340 and 445 nm, respectively.

![Absorption spectrum of Cu-Zn RNA polymerase (RPase).](image)

**Effect of RNA polymerase, template, and substrates on the corrected emission spectra of (5'-AmNS)UpA.** The concentrations of (5'-AmNS)UpA and RNA polymerases were 0.1 and 20 μM, respectively, in 80 mM Tris-Cl (pH 8) buffer containing 10 mM MgCl₂, 50 mM KCl, and 2 mM DTT. Curve a, (5'-AmNS)UpA alone; curve b, (5'-AmNS)UpA + Zn-Zn RNA polymerase; curve c, (5'-AmNS)UpA + Co-Zn RNA polymerase; curve d, (5'-AmNS)UpA + Co-Zn RNA polymerase + poly(dA-dT) (25 μg/ml); curve e, (5'-AmNS)UpA + Co-Zn RNA polymerase + poly(dA-dT) + UTP (0.4 mM) + ATP (0.4 mM).

![Spectral overlap between the absorption spectrum of Co-Zn RNA polymerase (RPase) and the corrected emission spectra of (5'-AmNS)UpA and (γ-AmNS)UTP.](image)
Intrinsic Metal Ion-Substrates Distances of RNA Polymerase

### TABLE I

Relative fluorescence, quenching (Q), and distances (r) from cobalt on the β subunit of Co-Zn RNA polymerase (RPase) to the elongator, (γ-AmNS)UTP

<table>
<thead>
<tr>
<th>RPAse (25 μM)</th>
<th>Poly(dA-dT)* (54 nM)</th>
<th>UTP (0.4 mM)</th>
<th>ATP (0.4 mM)</th>
<th>Relative fluorescence</th>
<th>Q (%)</th>
<th>r Å</th>
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<tr>
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<td>-</td>
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* 700 base pairs in length.

### TABLE II

Relative fluorescence, quenching (Q), and distances (r) from cobalt on the β subunit of Co-Zn RNA polymerase (RPase) to the elongator, (γ-AmNS)UTP

(γ-AmNS)UTP (0.1 μM) was present in all samples. The errors of average distance measurements were estimated to be within ±5%. R0 value for the (γ-AmNS)UTP and Co-Zn RNA polymerase pair in the absence of template was measured to be 21.3 Å. The buffer used was 80 mM Tris-Cl (pH 7.9), 2 mM p-mercaptoethanol, 10 mM MgCl2, 50 mM KCl, and 2 mM DTT.

<table>
<thead>
<tr>
<th>RPAse (25 μM)</th>
<th>Poly(dA-dT)* (54 nM)</th>
<th>UpA (0.4 mM)</th>
<th>Relative fluorescence</th>
<th>Q (%)</th>
<th>r Å</th>
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* 700 base pairs in length.

### DISCUSSION

In our previous communication (21), we demonstrated that (γ-AmNS)UTP is capable of initiating RNA synthesis in the presence of poly(dA-dT) as DNA template. Moreover, we have confirmed the earlier reports (22, 35) that the mononucleoside triphosphate fluorescent analogs, (γ-AmNS)UTP and (γ-AmNS)ATP, could be synthesized and incorporated into RNA product with about 60% efficiency of the parent NTPs by E. coli RNA polymerase.

We have developed a new procedure to selectively substitute the zinc ion in the β subunit of E. coli RNA polymerase with another metal ion under non-denaturation conditions. The intrinsic metal ions of RNA polymerase can be removed by the inactivation of the enzyme in the presence of a metal chelator (DCTA), an oxidizing agent (NaNO3), and a sulfur-containing reducing agent such as DTT. The similar reagents have been employed in other enzyme systems (30, 39). A plausible mechanism for the inactivation of enzyme and the removal of intrinsic metal ions from E. coli RNA polymerase is the reconstitution of metal hybrid RNA polymerases obtained by this method are higher than those of the corresponding hybrid RNA polymerases obtained via urea denaturation or low pH treatment methods (18, 40). This may be attributed to the fact that there are no severe changes in the hydrophobic region or charged parts of the protein as may be the case in the other two methods.
It has been known that both tetrahedral \((T_d)\) and octahedral geometries of high spin \(\text{Co}^{II}\) result in two absorption bands with similar intensity in the region of 500 nm \((41, 51)\). However, the \(T_d\) cobalt complexes commonly give rise to peaks of lower intensity near 700 nm, and the five coordinated \(\text{Co}^{II}\) complexes also exhibit bands throughout the 450–700 nm region \((43, 44)\). The spectral intensity, therefore, is a more sensitive and appropriate indicator of geometry. The molar absorptivity \(\epsilon\) of \(T_d\) \(\text{Co}^{II}\) is 100-fold greater than that of octahedral \(\text{Co}^{II}\) \((43)\). The most intensive visible absorption bands in the distorted \(T_d\) \(\text{Co}^{II}\) complexes generally have an \(\epsilon\) value exceeding 250 m\(^{-1}\) cm\(^{-1}\), while five coordinated \(\text{Co}^{II}\) complexes have \(\epsilon\) between 50 and 225 m\(^{-1}\) cm\(^{-1}\) \((41–44)\). In our earlier studies, the \(\epsilon\) of \(\text{Co-Co RNA polymerase}\) was found to be about 250–350 m\(^{-1}\) cm\(^{-1}\) in the 500–700 nm region \((45)\) corresponding to the spin-allowed ligand field transition \(\Delta_A^e \rightarrow T_d(P)\) \((46)\). The position and intensities of these bands are indicative of a distorted \(T_d\) high spin \(\text{Co}^{II}\) \((46–48)\). The \(\epsilon\) value of \(\text{Cu-Zn RNA polymerase}\) and \(\text{Co-Zn RNA polymerase}\) is 815 m\(^{-1}\) cm\(^{-1}\) \((637 \text{ nm})\) and 2589 m\(^{-1}\) cm\(^{-1}\) \((465 \text{ nm})\), respectively \((\text{Figs. } 2 \text{ and } 3)\). The intense band around 350–395 nm for \(\text{Co-Zn RNA polymerase}\) is very likely due to an \(S^+ \rightarrow \text{Co}^{II}\) charge transfer absorption, in accord with those observed in \(\text{Co}^{II}\)-thiolate complexes \((49)\). In each of the above complexes, at least one thiolate ligand was coordinated to the cobaltous ion. The calculated \(\epsilon\) values were in the range of 900–1300/Co–S-Cys bond. Thus, in the case of \(\text{Co-Zn RNA polymerase}\), \(\epsilon = 2709 \text{ m}^{-1} \text{ cm}^{-1}\) at 395 nm is consistent with the conclusion that a total of two or three Cys–S groups may be involved in the coordination of one cobalt atom. The assignment of the absorption band at 465 nm is still uncertain. Tentatively, if the charge-transfer bands at 395 and 465 nm of \(\text{Co-Zn RNA polymerase}\) correspond to those of iron complexes at 365 and 495 nm, respectively, then the energy separation between two charge-transfer bands in \(\text{Co-Zn RNA polymerase}\) of 3811 cm\(^{-1}\) is in good agreement with the 3696 cm\(^{-1}\) energy separation previously observed in the Fe–S bond of Fe-rubredoxin \((48, 50)\). A rough correlation between the charge-transfer band position and coordination geometry around metal ions has been observed for a series of metal ions possessing a given ligand environment \((50)\). The correlation further supports our earlier contention that cobalt in Co-Zn RNA polymerase is in distorted \(T_d\) geometry. The difference absorption spectrum of Co-Co RNA polymerase \((51)\) which exhibits weak negative peaks at 375 and 356 nm and a positive one at 343 nm resembles the CD spectrum of Co–S–Cys in Co-rubredoxin \((52)\) which exhibits a strong negative extremum at 375 and 346 nm and a positive extremum at 322 nm. The ligand geometry in Co–S–Cys complexes \((52)\) was proposed to be a distorted \(T_d\) with four cysteine groups coordinated to one cobalt. The shift of CD extrema from 322 nm to the absorption peak at 343 nm in Co-Co RNA polymerase may explain a possible coordination of three cysteine residues along with one histidine residue which brought about the lower energy absorption. This reasoning is further supported by our recent chemical modification studies of \(E. coli\) RNA polymerase with diethylpyrocarbonate \((53)\). Our results suggest that histidine residues may be involved in the coordination of intrinsic metal ions. Finally, the 637 nm \(\text{ (i.e. } 15,698 \text{ cm}^{-1}\text{)}\) absorption band of \(\text{Cu-Zn RNA polymerase}\) is in reasonable agreement with the intense band of 13,000–16,000 cm\(^{-1}\) for the blue copper proteins \((54)\). This latter band was assigned to be a charge-transfer absorption, suggesting a distorted \(T_d\) coordination around the copper.

The fluorescent nucleotide analogs, \((5'\text{-AmNS})\text{UpA}, (\gamma'\text{-AmNS})\text{UTP}, \text{ and } (\gamma'\text{-AmNS})\text{ATP}, \text{ all have absorption maxima between } 300 \text{ and } 360 \text{ nm which are well resolved from the absorption bands of proteins and nucleic acids. This allows a selective excitation of the fluorophore at } 340 \text{ nm and alleviates possible inner filter effects resulting from the absorption by proteins or nucleic acids. Since their fluorescence emission spectra in the near visible region overlaps the ab-
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Absorption band of Co-Zn RNA polymerase, they are excellent fluorescent probes to study the interactions of metal hybrid RNA polymerase with dinucleotide and mononucleotide bound at the initiation and elongation sites, respectively. The spectral characteristics of the energy donor (fluorescent analog) and the energy acceptor (cobalt in Co-Zn RNA polymerase) allow the determinations of the distances between the metal and the substrate binding sites in the β subunit of E. coli RNA polymerase using the FRET technique.

The distance from the initiator, (5'-AmNS)UpA, to the cobalt ion in Co-Zn RNA polymerase was measured to be 17.4 Å in the absence of template by FRET. While the fluorescence of (5'-AmNS)UpA was quenched by Co-Zn RNA polymerase, the addition of template causes a further ~1% quenching and 0.2 Å decrease in distance, which may explain the tightening of the substrate-enzyme complex in the presence of the template. Upon addition of UTP, it results in the formation of (5'-AmNS)UpAPU with concurrent enhancement of fluorescence which may be due to the increased freedom of movement for the naphthalene ring around the 5' end of RNA product. The naphthalene ring may rotate and float at the 5' end of RNA product during the elongation of RNA chain, consistent with the additional enhancement of fluorescence observed upon further addition of ATP. The distance from (γ-AmNS)UTP bound at the elongation site to the cobalt ion in Co-Zn RNA polymerase was similarly measured to be 17.5 Å in the absence of template. The addition of template causes a further 2.3% quenching in the fluorescence of (γ-AmNS)UTP. The enhancement of the fluorescence upon the addition of initiator, UpA, signals the breakage of the α-β phosphodiester bond and the release of fluorophore, (γ-AmNS)PP, which has a higher quantum yield than the starting substrate (35). A schematic representation of the spatial relationship between the cobalt in Co(β)-Zn(β') RNA polymerase and the initiator, (5'-AmNS)UpA, or the elongator, (γ-AmNS)UTP, in the presence of poly(dA-dT) is depicted in Fig. 6. This indicates that AmNS, at the 5' end of UpA, is lying above the plane of adenine ring and may float freely, giving rise to the fluorescence enhancement. The distances from the center of the fluorophore AmNS to the 6-amino and N° of adenine or N° of uridine rings are calculated to be approximately 15 Å. If one subtracts the distances from Co(II) to the initiation (17.4 Å) or to the elongation site (17.5 Å), the net distances correlate well with the conformation of a coordinated Co(II)-ATP complex (55-58).

The calculated distances between cobalt and the initiation or the elongation site by FRET studies can be compared with the distance between the initiation and elongation site obtained by EPR alone (59) and with that between cobalt and elongation site by the combined EPR and FRET measurements as indicated below.

The rate constants ($k_r$) for the energy transfer from (γ-AmNS)ATP to Zn-Zn RNA polymerase or Co-Zn RNA polymerase were calculated to be in the range of 10^6 s⁻¹, and the $k_r$ value is 7-fold greater for Co-Zn RNA polymerase than for Zn-Zn RNA polymerase. Furthermore, we have observed that the phosphodiester bond formation between UpA and (γ-AmNS)UTP catalyzed by Zn-Zn or Co-Zn RNA polymerase resulted in fluorescent enhancement. These facts enable us to further study the catalytic processes and the spatial relationship between the intrinsic metal ion and substrate binding sites of RNA polymerase by fast kinetic techniques. The stopped flow studies utilizing these fluorescent substrate analogs and metal hybrid enzymes are in progress.
REFERENCES