Direct Coordination of Nucleotide with the Intrinsic Metal in *Escherichia coli* RNA Polymerase. A Nuclear Magnetic Resonance Study with Cobalt-Substituted Enzyme†

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ABSTRACT: Nuclear magnetic resonance studies were performed with *Escherichia coli* RNA polymerase (RPase) in which one of the two intrinsic Zn ions was substituted with Co(II) ion (Co–Zn RPase). The Co ion was located in the β subunit which contains the initiation site of the enzyme. The paramagnetic effect of Co–Zn RPase on the relaxation rates of rapidly exchanging water protons indicated that the Co ion was accessible to solvent. There were approximately two water molecules in the inner coordination sphere of the Co ion, one of which could be replaced by the substrate adenosine 5′-triphosphate (ATP) or the initiator adenylyl-(3′→5′)-adenine (ApA) but to a much less extent by uridine 5′-triphosphate. The effects of ATP and ApA did not require the presence of DNA or Mg(II) ions, and their K_d values were estimated to be 0.15 and 0.075 mM, respectively. These results showed that the Co ion was at the initiation site. From the measurements of the paramagnetic effects of Co–Zn RPase on the relaxation rates of ^1H and ^{31}P nuclei of ATP, the distances from the intrinsic Co ion to H_2, H_8, and $H_{1'}$ were determined to be 4.1 ± 0.6, 3.6 ± 0.5, and 6.8 ± 0.8 Å, respectively, and those to the α-, β-, and γ-phosphorus atoms were 10.5 ± 0.7, 15.1 ± 1.1, and 14.1 ± 0.8 Å, respectively. These spatial relationships clearly indicate that the Co ion is direcly coordinated to the base moiety of ATP bound at the initiation site. Thus, the intrinsic metal in the β subunit of RNA polymerase may play a regulatory role in the recognition of the initiating nucleotide and may orient the nucleotide in a stereospecific position for the initiation.

The existence of intrinsic Zn ions in prokaryotic and eukaryotic RNA polymerase (RPase)[1] has been established [see review by Wu & Wu (1981)], yet the mechanistic role of these metal ions is not known and has been a subject of active investigation in recent years. *Escherichia coli* RNA polymerase contains two intrinsic Zn ions, one of which is located in the β subunit and the other in the β' subunit (Wu et al., 1977; Miller et al., 1979). To investigate the structural and functional role of these intrinsic metal ions, we have previously prepared by biosynthetic incorporation in vivo the enzyme in which both of the Zn ions were substituted with Co(II) ions (Speckhard et al., 1977). Recently, we have developed an in vitro method to substitute the intrinsic Zn ion in the β subunit with various divalent metal ions such as Co(II), Mn(II), Ni(II), and Cu(II) (Chatterji & Wu, 1982). While Co–Co RPase is enzymatically as active as Zn–Zn RPase, the specific activities of metal hybrid RPases vary in the order Zn–Zn ≈ Co–Zn ≈ Mn–Zn > Ni–Zn > Cu–Zn. Comparative studies of the biochemical properties of these metal-substituted RPases suggest that the intrinsic metals may play a role in RNA chain initiation and promoter recognition (Speckhard et al., 1977). Physically, all the metal-substituted enzymes are similar except that Co–Co, Co–Zn, and Ni–Zn RPases exhibit characteristic absorption spectra in the visible region. These spectra can be perturbed by addition of nucleoside triphosphates or template analogues, indicating that the intrinsic metal ions, particularly the one in the β subunit, may be involved in substrate or template binding. It it not clear, however, whether the intrinsic metal ions interact directly with substrate or template. To answer this question, it is necessary to determine the distance between the metal and substrate (or template) binding sites on the enzyme.

Because Co(II) is paramagnetic, the distances between the intrinsic metal ion and the enzyme-bound substrate (or template) molecule can in principle be determined in Co–Zn RPase by NMR spectroscopy. In the past decade, the nuclear magnetic relaxation method, which determines the distances from the individual atoms (^1H, ^{13}C, and ^{31}P) of a molecule to a paramagnetic reference point ≤24 Å away, has emerged as a useful approach to the study of the conformation and arrangement of enzyme-bound substrate in solution (Mildvan & Cohn, 1970; Mildvan & Gupta, 1978). Using extrinsic Mn(II) and Cr(III) complexed to nucleotides as paramagnetic probes, Mildvan and his colleagues (Bean et al., 1977; Stein & Mildvan, 1978) have applied the NMR technique to study the geometry and conformation of nucleotides bound to *E. coli* RNA polymerase. In this paper, we have investigated the paramagnetic effects of intrinsic Co(II) ion in Co–Zn RPase on the ^1H relaxation rates of water in the presence of the substrate ATP to calculate the distances from the paramagnetic probe to various substrate nuclei. These distances are then used to determine the conformation and arrangement of the bound substrate with respect to the Co(II) ion, which should provide meaningful clues to the mechanism of gene

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; ApA, adenylyl-(3′→5′)-adenine; UpA, uridylyl-(3′→5′)-adenine; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; UTP, uridine 5′-triphosphate; RPase, RNA polymerase; Zn–Zn or Co–Co RPase, RNA polymerase containing two intrinsic Zn or Co ions, respectively; Co–Zn, Mn–Zn, Ni–Zn, or Cu–Zn RPase, RNA polymerase containing one intrinsic Zn ion and one intrinsic Co, Mn, Ni, or Cu ion, respectively; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; HPLC, high-performance liquid chromatography.

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transcription and the role of intrinsic metal in RNA polymerase.

Experimental Procedures

Materials. *E. coli* RNA polymerase was purified by the method of Burgess & Jendrisak (1975) from *E. coli* cells purchased from Grain Processing Co., Muscatine, IL. The purified enzyme was at least 95% pure as judged by acrylamide gel electrophoresis. Co–Zn core polymerase was obtained from in vitro substitution as described in the previous paper (Chatterji & Wu, 1982). The enzyme was assayed for its activity at the conclusion of each NMR experiment, and it retained a minimum of 80% of its initial activity. Adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) of HPLC grade were purchased from ICN. The dinucleoside monophosphate ApA was obtained from P-L Biochemicals. For NMR studies, all nucleotides were dissolved in 100% D_2O, and their pDs were adjusted to 7.5. D_2O was a product of Stohler Isotope Chemicals. Nucleotide concentrations were determined by the UV absorption at 259 nm for ATP ($\epsilon = 1.54 \times 10^4$ mmol^{-1} cm^2), at 262 nm for UTP ($\epsilon = 1 \times 10^4$ mmol^{-1} cm^2) (CRC Handbook of Biochemistry, 1973), and at 257 nm for ApA ($\epsilon = 2.78 \times 10^4$ mmol^{-1} cm^2) (Ts'o et al., 1966). All other reagents were the highest grade obtainable. Trace metal contaminants were removed from buffers, enzyme, and nucleotide solutions by passing through Bio-Rad Chelex-100 columns.

Preparation of Enzyme Solutions for NMR Studies. The core Co–Zn RNA polymerase (5 mg/mL), which was stored at −20 °C in 10 mM Tris-HCl (pH 8) containing 60% glycerol (v/v), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 M KCl (buffer I), was precipitated by adding solid $(NH_4)_2SO_4$ (0.45 g/mL) together with 1 N NaOH (4 μL/mL) and centrifuged in a Sorvall SS34 rotor for 20 min at 15 000 rpm. The pellet was resuspended in 10 mM Tris-HCl (pD 7.5) and 0.15 M KCl (buffer II) and passed through a Sephadex G-25 column equilibrated with the same buffer. The protein fractions were combined and concentrated by vacuum dialysis against deuterated buffer II (H_2O was replaced by D_2O) to a final concentration of 20 mg/mL. Dilution of the enzyme solution to a proper concentration for NMR measurements was done with deuterated buffer II in the presence or absence of 10 mM $MgCl_2$. The concentrations of Co–Zn core RPase used were from 5×10^{-6} to 2×10^{-5} M.

Magnetic Resonance Measurements. Proton magnetic resonance spectra were obtained at 80 MHz with a Varian CFT-20 Fourier-transform NMR spectrometer and at 360 MHz with a Bruker WH-360 spectrometer. The latter equipment was located at the Brookhaven National Laboratory, Upton, NY. Water proton relaxation rates at 24 and 40 MHz were measured by using standard pulsed methods in an NMR spectrometer located at the University of Pennsylvania, Philadelphia. The longitudinal relaxation time of water proton in 1 mM $MnCl_2$ solution was used as a standard in these measurements. Measurements of ^{31}P NMR spectra at 40.5 MHz were made by using a Varian XL-FT-100 spectrometer. The longitudinal relaxation rates ($1/T_1$) were measured by the inversion recovery technique or by using a PD–180°–τ–90° pulse sequence (Carr & Purcell, 1954). The transverse relaxation rates ($1/T_2$) were determined by measuring the line width at half-height in hertz, $\Delta\nu$, and applying the equation $1/T_2 = \Pi\Delta\nu$. The ribosyl proton spectra of the ATP molecule were obtained by nullifying the residual HDO resonance by using a water-eliminated Fourier-transform (WEFT) technique (Patt & Sykes, 1972; Mooberry & Krugh, 1975). Deuteration of ApA at the H_8 position was carried out as described previously (Ts'o et al., 1969). UV and ^1H NMR spectra showed that ApA was undegraded and selectively deuterated at H_8. The temperature for the relaxation measurements was 24 ± 1 °C.

Theoretical Basis for Calculations

The calculations in this study are based on the theory of Solomon and Bloembergen (Solomon, 1955; Solomon & Bloembergen, 1956; Bloembergen, 1957; Bernheim et al., 1959) for the paramagnetic effects on the nuclear spin relaxation rates of a magnetic nucleus which is bound near a paramagnetic species. The details of such calculations and their applications to the biological systems have recently been reviewed (Mildvan, 1977; Mildvan & Gupta, 1978; Mildvan et al., 1980).

In general, the paramagnetic effect of an enzyme-bound transition metal on the longitudinal relaxation rate ($1/T_{1p}$) of a nearby magnetic nucleus such as the ^1H or ^{31}P atom of a substrate which is exchanging into the enzyme–metal complex depends predominantly on four parameters: the lifetime of the complex (τ_M), the relative stoichiometry or coordination number of the substrate (q), the correlation time for electron–nuclear dipolar interaction (τ_c), and the distance from the paramagnetic metal to the substrate nucleus in the complex (r). The principle of the experiment is to calculate the distance by evaluating the other three parameters on the basis of the relationship

$$\frac{1}{fT_{1p}} = \frac{q}{T_{1M} + \tau_M} + \frac{1}{fT_{os}} \tag{1}$$

where f is the ratio of the concentration of the bound metal to the total concentration of substrate in solution, $1/T_{os}$ is the small outer-sphere contribution to the relaxation rate, and $1/T_{1M}$ is the relaxation rate of the substrate nucleus in the enzyme–metal–substrate complex. $1/T_{1p}$ can be obtained experimentally by subtracting the relaxation rate of a substrate nucleus measured in the presence of a diamagnetic metalloenzyme (Zn–Zn RPase) from that measured in the presence of a paramagnetic metal-substituted enzyme (Co–Zn RPase). The value of $1/T_{1M}$ is given by

$$\frac{1}{T_{1M}} = (C/r)^6[f(\tau_c)] + \frac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2 \frac{\tau_e}{1 + \omega_s^2\tau_e^2} \tag{2}$$

where C is a product of constants proportional to the spin state and average g value of the metal ion and normally equals 895 ± 180 for high-spin Co(II) (Mildvan et al., 1980), S is the electron spin quantum number, ω_s is the Larmor precession frequency for electron spin, τ_e is the correlation time for the scalar or contact interaction, and $A/\hbar$ is the electron–nuclear hyperfine coupling constant. The correlation function, $f(\tau_c)$, can be expressed as

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2\tau_c^2} \tag{3}$$

where ω_I is the Larmor precession frequency for nuclear spin at a given magnetic field. The correlation time, τ_c, is given by

$$1/\tau_c = 1/\tau_s + 1/\tau_M + 1/\tau_R \tag{4}$$

where τ_R is the tumbling time of the enzyme–metal or enzyme–metal–substrate complex and τ_s is the electron spin relaxation time. The value of τ_c may be frequency dependent

Table I: Effect of Co–Zn Core RPase on the Longitudinal Relaxation Rates of the Water Proton and the Correlation Times (τ_c) at Various Frequencies[a]

frequency (MHz)	$1/[T_1(\text{Co})]$ (s^{-1})	$1/[T_1(\text{Zn})]$ (s^{-1})	$1/T_{1p}$ (s^{-1})	$1/(fT_{1p})$ (s^{-1})	τ_c (s)[b]
24	0.39	0.26	0.13	3.61×10^5	9.52×10^{-12}
40	0.35	0.26	0.09	2.50×10^5	9.52×10^{-12}
80	0.28	0.21	0.07	1.78×10^5	9.53×10^{-12}
360	0.26	0.20	0.06	1.69×10^5	9.79×10^{-12}

[a] The concentration of the Co–Zn core RPase used was 2×10^{-5} M in all cases. The error for the measurement of $1/T_1$ in each case was estimated to be within 5%. [b] The values of τ_c at different frequencies were calculated from eq 5 by using the best-fit values of $B = 3.43 \times 10^{23}$ s^{-2} and $\tau_\nu = 6.12 \times 10^{-14}$ s obtained by computer search (see text).

since it is often dominated by τ_s, which disperses with frequency according to the equation

$$\frac{1}{\tau_s} = B\left(\frac{\tau_\nu}{1 + \omega_s^2\tau_\nu^2} + \frac{4\tau_\nu}{1 + 4\omega_s^2\tau_\nu^2}\right) \quad (5)$$

where B is a constant dependent on the zero-field splitting at the paramagnetic center and τ_ν is the time constant for distortion of this symmetry (Bloembergen & Morgan, 1961).

In eq 2, the first term represents the contribution of electron nuclear dipole–dipole interaction to the relaxation rate, and it is this term that makes it possible to calculate the distance between the nuclei of a ligand and the paramagnetic metal center. The second term represents the scalar or contact contribution to the relaxation rate. With the assumption that (a) the scalar contribution in eq 2 is negligible, (b) the outer-sphere contribution is small, and (c) the substrate is in rapid exchange with the enzyme–metal–substrate complex, then $fT_{os} \gg fT_{1p} \gg \tau_M$, and eq 1 can be approximated to

$$\frac{1}{fT_{1p}} = \frac{q}{T_{1M}} = q(C/r)^6[f(\tau_c)] \quad (6)$$

Combination of eq 3 and 6 gives

$$r = C\left[qfT_{1p}\left(\frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2\tau_c^2}\right)\right]^{1/6} \quad (7)$$

There are three unknowns in eq 7: r, q, and τ_c. The correlation time τ_c can be determined by various ways, but the frequency dependence of $1/T_{1p}$ is the most convenient one, requiring few ancillary assumptions. The remaining two unknowns, q and r, cannot be separately evaluated by measurement of $1/fT_{1p}$ alone. An independent determination of the relative stoichiometry of the substrate in the enzyme–metal–substrate complex (q) by titrating the metal-substituted enzyme with substrate measuring the relaxation rate of water proton (PRR titration) will permit a calculation of the distance r according to eq 7.

Results

Effect of Co–Zn RPase on ^1H Relaxation Rates of Water. The Co–Zn RPase core enzyme was used in all NMR experiments described below, although essentially the same results were obtained with the holoenzyme. The Co–Zn RPase increased the longitudinal relaxation rate ($1/T_1$) of water protons by an amount significantly greater than did an equal concentration of native Zn–Zn enzyme. The paramagnetic contribution of the enzyme-bound Co(II) on the longitudinal relaxation rate of water protons ($1/T_{1p}$) was calculated according to

$$1/T_{1p} = 1/[T_1(\text{Co})] - 1/[T_1(\text{Zn})] \quad (8)$$

where $1/[T_1(\text{Co})]$ and $1/[T_1(\text{Zn})]$ are the relaxation rates of water protons measured in the presence of Co–Zn and Zn–Zn RPases, respectively. The detectable paramagnetic effect on the $1/T_1$ of the water proton indicates that the intrinsic Co ion is accessible to solvent. In addition, a decrease in $1/T_{1p}$ was observed upon titrating the bound water on the 20 μM Co–Zn enzyme with various concentrations of UTP, ATP, or ApA, in the absence of DNA template and exogenous Mg(II) ions (Figure 1). The effect of UTP was much less than that of ATP or ApA. These results suggest that nucleotide substrate decreased the access of water protons to the intrinsic Co ion and that the nucleotide or initiator (ATP or ApA) with higher affinity for the initiation site exerts a larger effect than that (UTP) with lower affinity. From the curves shown in Figure 1, the K_d values for ATP and ApA in the enzyme–substrate complex were estimated to be 150 and 75 μM, respectively.

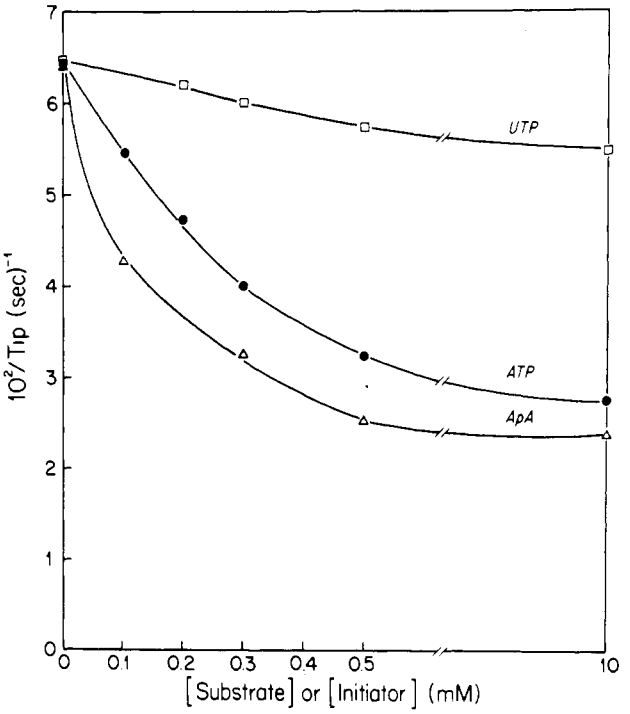

FIGURE 1: Effects of substrate (UTP or ATP) and initiator (ApA) binding on the relaxivity of water protons at 80 MHz in the presence of Co–Zn core RPase (2×10^{-5} M) in deuterated buffer II: UTP (□); ATP (●); ApA (△).

Determination of Correlation Times for the Co(II)–Proton Interactions. The correlation times for the dipolar effects of Co–Zn RPase on the protons of water and ATP were evaluated by examining the frequency dependence of $1/(fT_{1p})$. Table I summarizes the effect of Co–Zn RPase on the water proton longitudinal relaxation rates at 24, 40, 80, and 360 MHz. The observed frequency dependence of $1/T_{1p}$ indicates that the values of the relaxation rates are not limited by water proton exchange (i.e., $T_{1M} \gg \tau_M$). Similar frequency dependence was also observed for the $1/(fT_{1p})$ measured at 80 and 360 MHz

Table II: Effect of Co–Zn Core RPase on the Relaxation Rates of ATP Protons[a]

	80 MHz				360 MHz			
proton	$1/[T_1(Co)]$ (s^{-1})	$1/[T_1(Zn)]$ (s^{-1})	$1/T_{1p}$ (s^{-1})	$1/(fT_{1p})$ (s^{-1})	$1/[T_1(Co)]$ (s^{-1})	$1/[T_1(Zn)]$ (s^{-1})	$1/T_{1p}$ (s^{-1})	$1/(fT_{1p})$ (s^{-1})
H_8	12.50	2.32	10.18	1.02×10^4	11.11	2.27	8.84	8.80×10^3
H_2	7.14	2.13	5.01	5.01×10^3	6.06	1.82	4.24	4.24×10^3

[a] The concentration of the Co–Zn core RPase was 1×10^{-5} M, and that of ATP was 1 mM in deuterated buffer II at 24 °C. The error for the $1/T_1$ measurement was estimated to be within 5% as obtained by the least-squares analysis of the data. Each value of $1/T_1$ given represents the average of at least three independent measurements.

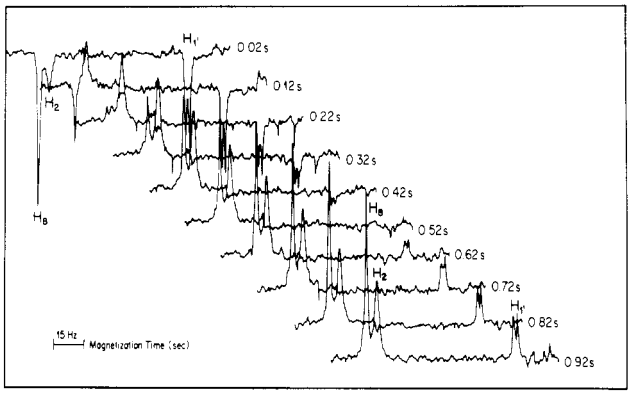

FIGURE 2: Longitudinal magnetic relaxation spectra of the proton resonance of ATP (10 mM) in a complex with Co–Zn core RPase (5×10^{-6} M) in deuterated buffer II at 80 MHz. The magnetization time is indicated at the right end of each spectrum.

for the protons of ATP in a complex with Co–Zn RPase (Table II), typical longitudinal magnetic relaxation spectra of which are shown in Figure 2. Moreover, as will be shown later (Table V), the displacement of ATP from Co–Zn RPase by ApA resulted in a large decrease in the $1/T_{1p}$ value of ATP protons, indicating that the outer-sphere contribution to $1/T_{1p}$ was small. Thus, the assumption made for eq 6 that $fT_{os} \gg fT_{1p} \gg \tau_M$ appears to be valid.

From eq 6, it follows that the ratio of $f(\tau_c)$ obtained directly from the ratio of $1/(fT_{1p})$ measured experimentally at any two frequencies can be used to calculate τ_c by assuming that the value of τ_c is independent of frequency. The τ_c values calculated in this way ranged from 0.7×10^{-11} to 1.2×10^{-11} s for water and ATP protons (Table III). The small but significant difference of τ_c values obtained at various frequencies suggests that the τ_c may be slightly frequency dependent. If τ_c is mainly governed by τ_s, then τ_c would be a function of frequency (eq 5), and the plot of T_1 vs. ω_I^2 would not be linear (Mildvan & Cohn, 1970). Indeed, such a plot for the effect of Co–Zn RPase on the water proton relaxation rate was found to be nonlinear (not shown). Thus, the frequency-dependent τ_c ($\simeq \tau_s$) values were determined by varying the values of τ_v and B in eq 5 in a computer search until the measured and calculated ratio of $1/(fT_{1p})$ gave the best agreement at all frequencies. The best-fit values obtained were $B = 3.43 \times 10^{23}$ s^{-2} and $\tau_v = 6.12 \times 10^{-14}$ s. Using these parameters, we calculated the actual values of τ_c at various frequencies according to eq 5 and included these values in Table I. As expected, there is only a very small dispersion of τ_c due to the frequency. Furthermore, the similarity of τ_c to $1/\omega_s$ ($\sim 10^{-11}$ s) rather than to $1/\omega_I$ ($\sim 10^{-9}$ s) agrees with the short electron spin relaxation time (5×10^{-13} to 5×10^{-11} s) generally observed for the high-spin Co(II) complex (Mildvan et al., 1980). The fact that no EPR spectrum was detectable for Co–Co RPase at 77 K (F. Y.-H. Wu and D. C. Speckhard, unpublished results) is also consistent with a high-spin Co(II) complex.

Table III: Ratios of $1/(fT_{1p})$ Measured at Various Frequencies and Values of τ_c Calculated by Assuming Its Independence of Frequency

	frequency (MHz)		$\frac{(fT_{1p})_{\nu_1}^{-1}}{(fT_{1p})_{\nu_2}^{-1}}$	$10^{11}\tau_c'$ (s)[a]
proton	ν_1	ν_2		
H_2O	24	40	1.44	0.9
	24	80	1.86	0.7
	40	80	1.29	1.2
	80	360	1.17	1.0
ATP H_8	80	360	1.15	1.1
ATP H_2	80	360	1.18	1.0

[a] The values of τ_c' were calculated according to eq 3 and the relation $(fT_{1p})_{\nu_1}^{-1}/(fT_{1p})_{\nu_2}^{-1} = f(\tau_c)_{\nu_1}/[f(\tau_c)_{\nu_2}]$ by assuming that the correlation time is independent of frequency.

Table IV: Paramagnetic Effect of Co-Zn Core RPase on the Longitudinal ($1/T_{1p}$) and Transverse ($1/T_{2p}$) Relaxation Rates of ATP at 360 MHz[a]

proton	$1/T_{1p}$ (s^{-1})	$1/(fT_{1p})$ (s^{-1})	$1/T_{2p}$ (s^{-1})	$1/(fT_{2p})$ (s^{-1})
H_8	8.84	8.84×10^3	206.1	2.06×10^5
H_2	4.24	4.24×10^3	117.8	1.18×10^5

[a] The concentration of Co-Zn core RPase was 1×10^{-5} M, and that of ATP was 10 mM in deuterated buffer II. The values of $1/T_{1p}$ and $1/T_{2p}$ given in the table represent the average of at least three independent measurements. The error estimated for $1/T_{1p}$ is ±5%, and that for $1/T_{2p}$ is ±15%.

Determination of the Number of H_2O and ATP Molecules Coordinated to the Co(II) Ion. With the value of τ_c determined, the number of water molecules coordinated to the Co ion in Co–Zn RPase (q) can be calculated according to eq 6 by using the $1/T_{1p}$ values measured for the water proton (Table I). In this case, f = [Co(II)]/[water proton] = $2 \times 10^{-5}/55.5$ and r = 2.47–2.65 Å as determined by the X-ray crystallographic study of the tetrahedral complex of Co(II) with water ligand in the first coordination sphere (McCandlish et al., 1978). Using the mean values of r = 2.56 Å and C = 895 (Mildvan et al., 1980), we calculated the number of water molecules coordinated to the Co ion (q) to be 2.2 according to eq 6. Addition of ATP or ApA to the Co–Zn RPase solution reduced the $1/T_{1p}$ value of water to half (Figure 1), suggesting that one of the two Co-bound water molecules was replaced by the substrate ATP. From eq 6, it can be seen that the estimation of q may be subjected to a large uncertainty due to its proportionality to the sixth power of both C and r. Nevertheless, it appears that the q value estimated here is rather close to the actual value since it has been estimated that only one ATP molecule binds to the initiation site of RPase (Wu & Goldthwait, 1969) and that the Co ion in Co–Zn RPase is located at this site (see below).

Effect of Co–Zn RPase on ^1H Relaxation Rates of the Base Protons of ATP. The paramagnetic effects of Co–Zn RPase

Table V: Effect of ApA on the Longitudinal Relaxation Rates of H_8 of ATP Complexed with Co-Zn RPase[a]

[ApA] (mM)	$1/T_{1p}$ (s^{-1})
0	10.2
1	6.5
3	5.7
5	5.0
10	3.8
20	1.9

[a] The $1/T_{1p}$ value was determined at 80 MHz with 10 mM ATP complexed with 1×10^{-5} M Co-Zn RPase. So that interference with the H_8 signal of ATP could be avoided, ApA was deuterated at the H_8 position (see Experimental Procedures).

on the longitudinal ($1/T_{1p}$) and transverse ($1/T_{2p}$) relaxation rates of H_2 and H_8 of ATP (10 mM) at 360 MHz are given in Table IV. In both cases, $1/(fT_{2p})$ is more than an order of magnitude greater than $1/(fT_{1p})$. Since the largest value of $1/(fT_{2p})$ sets a lower limit to $1/\tau_M$, the pseudo-first-order rate constant for dissociation of the substrate from the enzyme–substrate complex, a value of $1/(fT_{1p})$ which is significantly (>5-fold) lower than that of $1/(fT_{2p})$ is indicative of ATP being rapidly exchangeable. This justifies our earlier assumption that the lifetime of the enzyme–substrate complex (τ_M) is short and its contribution to the T_{1p} measurements can be neglected.

The initiator ApA can be incorporated into the 5′ terminus of RNA product synthesized by RNA polymerase but is not the substrate for the elongation reaction (Downey & So, 1970; Downey et al., 1971). To test whether ATP binding to Co–Zn RPase is at the initiation site of the enzyme, we have measured the displacement of ATP from the enzyme–substrate complex by ApA. In these experiments, H_8 of ApA was converted to D_8 (Ts'o et al., 1969) to avoid the interference of an H_8 NMR signal of ATP by ApA. As shown in Table V, the relaxivity of H_8 of ATP in the presence of Co–Zn RPase can be quantitatively decreased by increasing the concentration of ApA. Most of the paramagnetic effect (>80%) on the relaxation of the ATP proton diminished by adding a large excess (20 mM) of ApA.

Effect of Co–Zn RPase on the ^1H NMR Spectra of the Sugar Protons of ATP. The effect of Co–Zn RPase on the ^1H NMR spectra of sugar protons was measured at 80 MHz by using the WEFT technique to remove the strong HDO signal which appeared around 5.5 ppm and overshadowed the $H_{2'}$ and $H_{3'}$ peaks of the sugar ring (Figure 3). The paramagnetic effect of $H_{3'}$ was greater than that of $H_{4'}$ or $H_{5'}$ as seen from the merger of two small peaks into a broad peak around 5.43 ppm. No NMR spectra for the OH groups on the sugar ring were visible, since the ATP solution was prepared in D_2O which converted OH into OD.

Effect of Co–Zn RPase on the ^{31}P Relaxation Rates of the Phosphorus Nuclei of ATP. We have also measured the paramagnetic effect of Co–Zn RPase on the longitudinal relaxation rates of the α-, β-, and γ-phosphorus nuclei of ATP at 40.5 MHz by ^{31}P NMR spectroscopy. The values of $1/T_{1p}$ for β-P and γ-P were very small, while that for α-P was slightly larger (Table VI). The values of $1/T_{2p}$ were also small, indicating a negligible scalar contribution (eq 2). These findings suggest that the phosphate groups are located far away from the paramagnetic metal center. With the assumption that the correlation time is frequency independent, τ_c for the Co(II)–^{31}P interaction is estimated by the ratio T_{1p}/T_{2p} (James, 1975) to be 1.8×10^{-11} s.

Calculations of the Distances from the Intrinsic Co(II) to ATP on Co–Zn RPase. The use of eq 7 to calculate distances

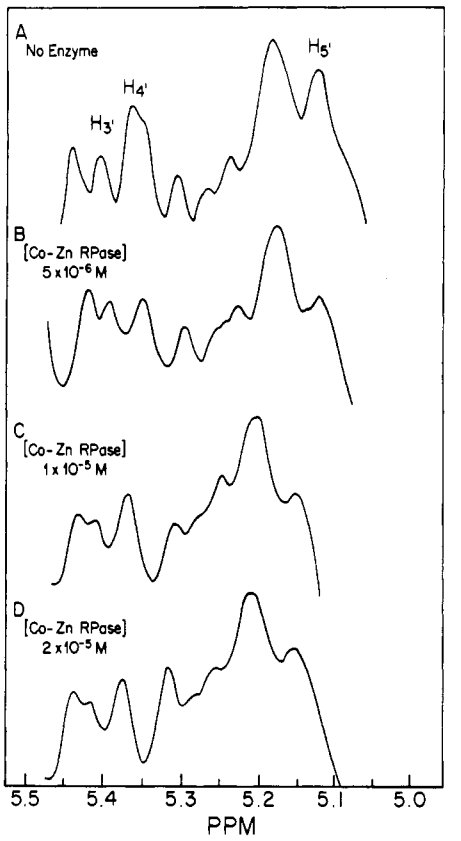

FIGURE 3: Proton NMR spectra of the ribosyl protons of 10 mM ATP in the absence (A) and presence of Co–Zn core RPase in deuterated buffer II at 80 MHz. The effects of the three following concentrations of enzyme were examined: 5×10^{-6} M (B); 1×10^{-5} M (C); 2×10^{-5} M (D).

Table VI: Calculation of the Distances from Co to the Protons and Phosphorus of ATP Complexed with Co–Zn Core RPase[a]

nucleus	$1/T_{1p}$ (s^{-1})	$1/(fT_{1p})$ (s^{-1})	r (Å)
H_8	10.18	10180	3.6 ± 0.5
H_2	5.01	5010	4.1 ± 0.6
$H_{1'}$	0.28	280	6.8 ± 0.8
α-P	0.017	17	10.5 ± 0.7
β-P	0.002	2	15.1 ± 1.1
γ-P	0.003	3	14.1 ± 0.8

[a] Components present were ATP (10 mM) and Co–Zn core RPase (1×10^{-5} M) in deuterated buffer II. The values of $1/T_{1p}$ were obtained at 80 MHz for ^1H and at 40.5 MHz for ^{31}P. The correlation times (τ_c) for all Co(II)–phosphorus interactions were estimated from the independent measurements of longitudinal ($1/T_{1p}$) and transverse ($1/T_{2p}$) relaxation rates of ATP phosphorus at 40.5 MHz and 24 °C. The error for ^{31}P NMR measurements was less than 5%.

from a paramagnetic center to an enzyme-bound ligand requires that $fT_{os} \gg fT_{1p} \gg \tau_M$ and that the $1/(fT_{1p})$ values not be limited by the rate of chemical exchange of the ligand out of the paramagnetic environment of the enzyme. As described above, these requirements are fulfilled for ATP on Co–Zn RPase. (Another assumption that the scalar contribution in eq 2 is negligible will be discussed later.) From an independent PRR titration, the number of ATP molecules coordinated to the intrinsic Co (q) was determined to be approximately 1 by estimating changes in the coordination number of rapidly exchanging water ligands as the substrate bound to the enzyme. In addition, the correlation times (τ_c) for the Co–^1H and Co–^{31}P interactions were estimated to be 1.0×10^{-11} and 1.8×10^{-11} s, respectively. With the values of q and τ_c

evaluated, the distances from the intrinsic Co to the protons (H_8, H_2, and $H_{1'}$) and to the α-, β-, and γ-phosphorus of ATP on Co–Zn RPase can be calculated by the measured $1/(fT_{1p})$ values. The calculated distances are listed in Table VI. The distances from Co to the base protons H_8 and H_2 are the shortest, being 3.6 ± 0.5 and 4.1 ± 0.6 Å from Co, whereas the α-, β-, and γ-phosphorus atoms are further away from Co with the distances of 10.5 ± 0.7, 15.1 ± 1.1, and 14.1 ± 0.8 Å, respectively.

It should be pointed out that a basic assumption in the application of the Solomon–Bloembergen method for distance calculation is that the isotropic hyperfine or scalar interaction is negligible; i.e., the second term in eq 2 can be neglected. This is not generally true for Co(II), particularly at short distances because the magnitude of the scalar contribution falls off rapidly with distance. Hence, the application to the three ^{31}P distances (10.5, 15.1, and 14.1 Å) is probably satisfactory, but one may question whether the distances of 4.1 and 3.6 Å to H_2 and H_8 are too small to assume a negligible scalar contribution. In order to test the importance of the scalar contribution, we have prepared Mn–Zn RPase [see Chatterji & Wu (1982)] in which a Mn(II) instead of a Co(II) ion is located at the initiation site of RPase and measured the distances from the Mn(II) ion to the H_2 and H_8 atoms of bound ATP by the same method. The preliminary results indicate that the distance to H_2 is 4.8 ± 0.5 Å and that to H_8 is 4.3 ± 0.4 Å, with the correlation time for the Mn–^{1}H interaction being 4×10^{-9} s (D. Chatterji and F. Y.-H. Wu, unpublished results). The values of these distances are within experimental error, in agreement with those obtained for Co–Zn RPase. Mn(II) has been widely used for the distance measurement by the Solomon–Bloembergen method because one can assume in almost all cases that the isotropic hyperfine or scalar contribution is negligible. Since the magnitude of the scalar contribution differs for Mn(II) and Co(II) due to different values of S and A, the distance values calculated from the dipolar term of eq 2 for Co–Zn RPase will be comparable to those for Mn–Zn RPase only if the scalar contribution to the relaxation process is small. Another possible source of error in distance calculations involving Co(II) is the assumption of an isotropic "g" tensor. However, as we could not detect any EPR spectra of Co–Zn RPase, the anisotropy in the g value could not be detected, and the constant C was taken to be 895 ± 180 (Mildvan et al., 1980) in our calculations.

Discussion

It has been suggested that the intrinsic Zn ions of *E. coli* RNA polymerase play a role in substrate or template binding (Speckhard et al., 1977; Wu et al., 1977). However, no evidence is available thus far concerning the spatial relationship between the intrinsic metal and substrate or template binding site on the enzyme. Recently, we have specifically replaced the Zn ion located in the β subunit of the enzyme by Co(II) and other divalent metal ions with an in vitro substitution method (Chatterji & Wu, 1982). In the present study, the observed paramagnetic effect of the Co–Zn RPase on the relaxation rates of fast-exchanging water protons indicates that this intrinsic Co ion is accessible to solvent. The number of fast-exchanging water protons in the coordination sphere of the Co(II) is approximately 2. Addition of a saturating concentration of ATP reduced this number to half of its original value, indicating that the substrate could replace or occlude the exchange of one of the two water ligands coordinated to the Co(II) in the enzyme. These results favor the idea that ATP is at the inner coordination sphere of the intrinsic Co(II) ion.

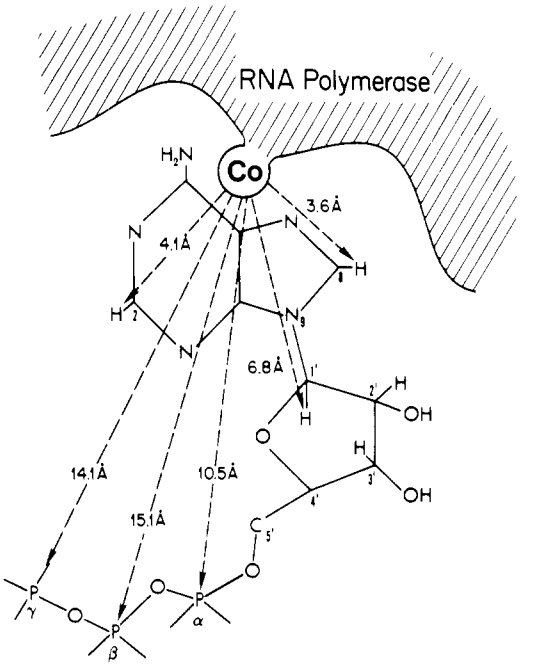

FIGURE 4: Distances from the intrinsic Co(II) to various proton and phosphorus nuclei of ATP on Co–Zn RPase.

That ATP is actually located at the inner coordination sphere is evident from the distance measurements. The measurements of the paramagnetic effect of Co–Zn RPase on the $1/T_{1p}$ of ATP permit us to estimate the distances from the intrinsic Co(II) to various proton and phosphorus nuclei of ATP on the enzyme. Based on the data given in Table VI, we have constructed a model illustrating the spatial relationship between the intrinsic Co and the bound ATP (Figure 4). The distance from Co(II) to H_8 and H_2 is 3.6 and 4.1 Å, respectively. These short distances strongly indicate the direct coordination of Co(II) to the base moiety of ATP, possibly through the unpaired electrons of N_7 and/or other nearby nitrogen atoms or formation of a π-type complex. The distances from Co(II) to the sugar proton $H_{1'}$ and the three phosphorus nuclei are too long for direct coordinations of the metal ion to these atoms. These larger distances are appropriate for a second sphere complex in which a coordinated water or other ligand may intervene between the metal and the substrate.

E. coli RPase was shown to possess a specific binding site for purine nucleotide in the absence of DNA template (Wu and Goldthwait, 1969). This site is called the initiation site because the initiation of RNA chains occurs primarily with purine nucleotides (Maitra & Hurwitz, 1965) and the interaction of nucleoside triphosphate at this site can be blocked by addition of rifampicin, an inhibitor of initiation. It has been proposed (Scrutton et al., 1971) that the initiation site of *E. coli* RPase may contain an intrinsic metal because the binding of a nucleotide to this site does not require extrinsic Mg(II) ions. In this paper, several lines of evidence have been presented to indicate that the intrinsic metal in the β subunit is, in fact, located at the initiation site: (1) The intrinsic Co(II) ion in the β subunit of Co–Zn RPase is coordinated directly to one molecule of purine nucleotide, ATP. This was demonstrated by the effects of Co–Zn RPase on the $1/T_{1p}$ values of various nuclei of ATP and on the $1/T_{1p}$ values of water protons in the presence of ATP. (2) The perturbations of NMR spectra of ATP by Co–Zn RPase do not require the presence of Mg(II) ion or DNA template. (3) The value of K_d for ATP in the enzyme–substrate complex was estimated

to be 0.15 mM by a PRR titration of Co–Zn RPase with various concentrations of ATP. Similar K_d values were reported for the binding of ATP to the initiation site as determined by equilibrium dialysis and fluorescence quenching techniques (Wu & Goldthwait, 1969). (4) The intrinsic Co appears to bind preferentially to purine nucleotide. UTP shows much less effect than ATP in both PRR titration and the perturbation of the visible absorption spectrum of Co–Zn RPase. (5) ApA, an initiator which does not serve as a substrate for RNA chain elongation, can displace quantitatively the interaction of ATP with the intrinsic Co(II) ion. (6) Finally, the fact that the intrinsic Co is located in the β subunit is in accord with our previous finding that this subunit contains the initiation site (Wu & Wu, 1974).

The precise function of the intrinsic metals in RNA polymerase has not been rigorously established. A number of functions can be visualized for the intrinsic Zn ions in transcription: (a) a catalytic role in the template and substrate binding or the subsequent phosphodiester bond formation; (b) a regulatory role in promoter recognition and specific initiation; and (c) a structural role in maintaining the proper conformation of the enzyme. Previous studies (Speckhard et al., 1977) have indicated that the intrinsic metal ions are involved in specific initiation of RNA chains. With T7 DNA as template, the Co–Co RPase is less efficient than the Zn–Zn enzyme in starting the RNA chains with GTP. The ratio of A starts to G starts is about 2 for Zn–Zn RPase, while it increases to 4–6 for Co–Co RPase. This difference can be explained by the direct participation of the intrinsic metal in binding the initiating nucleotide. As shown in Figure 4, the stacking of the intrinsic metal with the base moiety of the nucleotide at the initiation site may form a structural basis for the recognition of initiating substrate by the enzyme. Furthermore, the triphosphate group is positioned more than 10 Å away from the metal center, suggesting that it is not an essential part for the recognition. This is consistent with the previous finding that AMP, ADP, UpA, or ApA can also initiate the RNA chain (Downey & So, 1970; Downey et al., 1971).

In addition to the regulatory role, the intrinsic metal may play a role in catalytic function. It has been postulated (Mildvan & Loeb, 1979) that the intrinsic Zn coordinates to the 3′-OH group of the initiating nucleotide to facilitate the deprotonation of the 3′-OH, a necessary step for nucleophilic attack on the α-phosphorus of the incoming nucleotide to form the phosphodiester bond. Although the distance from Co(II) to 3′-OH was not determined in this study, it can been seen from the model in Figure 4 that this distance would be more than 7 Å. Thus, it appears unlikely that this intrinsic metal is participating directly in catalysis. Another possibility is that the intrinsic metal may coordinate to the 2′-OH group of ribonucleotide substrate. Such an effect may provide a basis for discrimination between 3′-deoxyribonucleotide and 2′-deoxyribonucleotide by RPase. Unfortunately, the effect of Co(II) on the $H_{2'}$ of ATP could not be measured since the HDO peak overlaps partially with the $H_{2'}$ peak which renders the interpretation of the spectra difficult.

Although the detailed structural information about the enzyme–substrate complex can be best answered by an X-ray crystallographic study, it is still impossible at the present time for RPase due to lack of a suitable crystal. By use of the NMR technique, we have mapped the geometry of the substrate coordinated to an intrinsic Co(II) ion at the initiation site which is located in the β subunit of *E. coli* RPase. Our data indicate that this intrinsic metal plays a recognition role in discriminating the initiating nucleotide. Furthermore, the metal interaction with the initiating substrate may orient the substrate in a stereospecific position suitable for catalysis. Thus, NMR studies appear to be very useful in providing further information on the role of intrinsic metals of RPase. It would be of great interest to examine the geometry of the enzyme-bound substrate in the presence of DNA template and to investigate the role of the other intrinsic metal located in the β' subunit which contains the template binding site. These studies which involve both Co- and Mn-substituted RPases are currently in progress in our laboratory.

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Fluorinated Ligands as Nuclear Magnetic Resonance Probes of Active-Site Nonequivalence in Abortive Ternary Complexes of Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The interactions of the dimeric horse liver alcohol dehydrogenase (LADH) with ligands in two nonreactive ternary complexes have been examined by using nuclear magnetic resonance and fluorescence techniques. One complex contains the enzyme, NADH, and the abortive alcohol substrate *p*-(trifluoromethyl)benzyl alcohol. The trifluoromethyl group allows the bound environment and bound to free exchange kinetics of this alcohol to be examined by using ^{19}F NMR. Binding isotherms of the coenzyme and the abortive substrate were examined by using fluorescence. Similar measurements were made with the enzyme, NADH, and the aldehyde analogue *p*-(trifluoromethyl)benzamide. For both complexes there was no evidence of cooperative equilibrium binding of any ligand. Careful measurements of the exchange kinetics of the fluorinated alcohol or amide when binding to the enzyme·NADH complex using NMR techniques showed that a single lifetime describes the exchange of ligands from both subunits of the protein. These results appear to rule out any site–site interactions in this system and support the notion that the observed biphasic kinetics observed in transient reactions of LADH with NAD and alcohols is a property of a single site in this system.

Horse liver alcohol dehydrogenase (LADH) is a dimeric zinc metalloenzyme which is one of the most thoroughly studied enzymes. However, the existence of intrasubunit communication or active-site nonequivalence within this dimer during catalysis has remained uncertain. Numerous reports have inferred half-of-the-sites reactivity (Bernhard et al., 1970; Dunn & Bernhard, 1971; McFarland & Bernhard, 1972; Luisi & Favilla, 1972; Luisi & Bignetti, 1974; Baici & Luisi, 1977; McFarland et al., 1977) or subunit interactions (Lindman et al., 1972; Dunn et al., 1979) within this system, while other reports either have found no evidence to support half-of-the-sites reactivity (Shore, 1969; Tatemoto, 1975; Hadorn et al., 1975) or have offered alternate explanations for data purporting to support a half-sited function for LADH (Pettersson, 1976; Kvassman & Pettersson, 1976; Weidig et al., 1977; Kordal & Parsons, 1979; Andersson & Mosbach, 1979).

We have recently examined half-inactivated LADH dimers for kinetic evidence of half-of-the-sites reactivity or subunit interactions. We also examined equilibrium binding isotherms and ligand desorption kinetics of LADH·NAD ternary complexes with trifluoroethanol or pyrazole for evidence of subunit interactions. These results were consistent with independent binding of ligands at each active site of the LADH dimer (Anderson & Dahlquist, 1982a,b).

In this report we have looked for equilibrium and kinetic evidence pertaining to subunit interactions in LADH by the study of complexes containing bound NADH in which turnover is not possible. Using ^{19}F NMR we are able to extend our earlier observations (Anderson & Dahlquist, 1980) and observe unique resonances for the substrate *p*-(trifluoromethyl)benzyl alcohol and the aldehyde analogue *p*-(trifluoromethyl)benzamide in complexes with LADH and NADH. Binding isotherms derived from ^{19}F NMR measurements and from protein NADH fluorescence quench measurements are consistent with independent ligand binding to each site of the dimer. We see only one type of bound environment in every LADH·NADH complex examined by ^{19}F NMR and only one desorption rate of bound *p*-(trifluoromethyl)benzamide. This suggests identical binding sites with identical ligand binding kinetics.

In competition experiments we have determined that the stability of alcohol in the LADH·NADH·alcohol complex is similar to that in the LADH·NAD·alcohol complex. This observation rules out mechanisms to explain the kinetic biphasicity observed during the corresponding aldehyde reduction that depend on an unusual kinetic or thermodynamic stability for this complex. Other mechanisms invoking unusual properties of LADH dimers with the abortive complex of NADH and alcohol bound at one site also appear inconsistent with our data.

Materials and Methods

Horse liver alcohol dehydrogenase was prepared as described in Anderson & Dahlquist (1979). Enzyme active site concentration was determined by the NAD–pyrazole assay of Theorell & Yonetani (1963) and is expressed in normality (*N*) to be distinguished from enzyme dimer concentration. β-NADH (grade III) was purchased from Sigma. *p*-(Trifluoromethyl)benzaldehyde was obtained from PCR, Inc.

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