Nuclear Magnetic Resonance Studies of Substrate Interaction with Cobalt Substituted Alcohol Dehydrogenase from Liver†

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ABSTRACT: The role of zinc in liver alcohol dehydrogenase has been studied by replacement of 1.3 and 3.5 of the four Zn(II) ions with Co(II) and measuring the effects of the paramagnetic Co(II) on the relaxation rates of the protons of water, ethanol, and isobutyramide. Water relaxation studies at 8, 24, 100, and 220 MHz indicate two classes of bound Co(II). The ~2 readily replaced Co(II) ions retain one fast exchanging water proton in their inner coordination spheres, while the ~2 slowly exchanging Co(II) ions coordinate no detectable water protons, indicating that the former replaced Zn(II) at the "catalytic sites" and the latter replaced Zn(II) at the "structural sites" detected crystallographically. Ethanol, acetaldehyde, and isobutyramide bind with appropriate affinities to the Co(II) substituted alcohol dehydrogenases decreasing the number of fast exchanging protons at the catalytic Co(II) site by ≥54%. Coenzyme binding causes smaller changes in the water relaxation rate which may be due to local conformation changes. The paramagnetic effects of Co(II) at the catalytic site on the relaxation rates of the methyl protons of isobutyramide at 100 and 220 MHz indicate that this analog binds at a site 9.1 Å from the catalytic Co(II). This distance decreases to 6.9 Å when NADH is bound, and a Co(II) to methyne proton distance of 6.6 Å is determined indicating a conformation change leading to the formation of a second sphere enzyme-Co(II)-isobutyramide complex in which a hydroxyl or water ligand intervenes between the metal and the substrate analog. Similar behavior is observed in the enzyme-ethanol complexes. The paramagnetic effects of Co(II), at the catalytic site, on the relaxation rates of the protons of ethanol at 100 and 220 MHz, indicate that this substrate binds at a site 12–14 Å distant from the catalytic Co(II) but that this distance decreases to 6.3 Å in the abortive enzyme-NADH-ethanol complex. The role of the catalytic Co(II) thus appears to be the activation of a hydroxyl or water ligand which polarizes the aldehyde carbonyl group by hydrogen bonding. The role of the structural Co(II), which is more distant from isobutyramide (9–11 Å), may be that of a template for protein conformation changes. By combining the present distances with those from previous magnetic resonance studies on the liver enzyme, the arrangement of coenzyme, metal, and substrate at the active site in solution can be constructed. This arrangement is consistent with that of ADP-ribose and zinc in the crystalline complex of liver alcohol dehydrogenase as determined by X-ray crystallography (Branden et al., (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2439).

The mechanistic role of the zinc ion in alcohol dehydrogenase has long been a subject of active investigation and discussion. While direct coordination of various electron rich atoms of the coenzymes NAD^+ and NADH by the zinc ion had long been proposed, kinetic and binding studies reviewed elsewhere (Mildvan, 1970) argue against such coordination. Thus removal of the four zinc atoms from alcohol dehydrogenase has little or no effect on the binding of coenzymes or coenzyme analogs (Weiner, 1969; Iweibo and Weiner, 1972). However, propinquity of two of the four zinc ions to bound coenzyme analogs on liver alcohol dehydrogenase has been suggested by recent X-ray (Branden et al., 1973) and electron spin resonance (EPR) data (Drott et al., 1974). Coordination of the substrate by the enzyme-bound zinc has been suggested (Abeles et al., 1957; Mildvan, 1970) and model studies have shown facilitation of hydride transfer to carbonyl groups by metal coordination of the latter (Shinkai and Bruice, 1973; Creighton and Sigman, 1971).

On the other hand, fluorimetric studies by Iweibo and Weiner (1972) showed that substrate competitive inhibitors form ternary complexes with the metal-free liver alcohol dehydrogenase and NADH. Also, in comparative studies of the binding of substrate competitive inhibitors to the native and cobalt-zinc mosaic enzymes, Young and Wang (1971) found no spectroscopic changes characteristic of coordination of such competitive inhibitors as azide to the catalytic class of metal ions.[1] These experiments suggest that the metal ion is not essential for the binding of substrate, although second sphere complexes are possible.

Recently, using magnetic resonance data from both yeast and liver alcohol dehydrogenase together with crystallographic data from the liver enzyme (Branden et al., 1973),

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[1] The term "catalytic" zinc ion was introduced by Drum and Vallee (1969b) to describe that class of metal ions which are most easily exchanged. The less easily exchanged zinc ions were termed "structural". Branden et al. (1973) used the same terminology to describe the Zn atoms near to and far from the bound ADP-ribose, respectively, in the crystallographic model. The present work establishes the consistency of this nomenclature.

Sloan and Mildvan (1974) suggested a model for the zinc-isobutyramide interaction involving coordination or at least propinquity of the substrate to the metal atom. In order to test this model more rigorously, we here employ nuclear relaxation techniques to determine the distances between the catalytic metals ions and the protons of isobutyramide and ethanol in both the partially (Young and Wang, 1971) and totally cobalt substituted liver alcohol dehydrogenase system (Drum and Vallee, 1970; Santiago and Shore, 1974). Determination of these distances has provided information on the positions of both the structural[1] and catalytic metal ions in relation to the substrate at the active site of alcohol dehydrogenase. Preliminary reports of this work have been presented (Sloan and Young, 1974; Sloan et al., 1974).

Experimental Section

Materials

Horse liver alcohol dehydrogenase, LADH,[2] purchased from Worthington Biochemicals (lot 2CA) for use in the metal exchange procedures was estimated from its specific activity to be at least 90% pure. Enzymatic activity assays followed the procedure of Drum et al. (1969a). The specific activity of the native zinc containing enzyme (Zn-ADH, 5.4-6.1 units/mg) was measured after dialysis at 4° against three changes of sodium phosphate buffer (0.1 *M*, pH 7.0) and then against a buffer (pH 7.0) containing the sodium salt of the anion to be used in the cobalt exchange experiments.

βNAD$^+$ and βNADH (Grade III) were purchased from Sigma Chemicals. Isobutyramide was an Eastman Organic reagent. Ethanol, a product of Publicker Industries, and acetaldehyde from Matheson Coleman and Bell were used without further purification.

Cobaltous chloride, a Baker Analytical reagent, and cobaltous sulfate and cobaltous acetate from Fisher were used as cobalt sources in metal exchange studies. Fisher certified atomic absorption reagents were used as standards in the metal analyses. All other chemicals employed were reagent grade.

Elution through Chelex 100 (Bio-Rad) was employed to free the buffer, coenzyme, and substrate solutions of metal contaminants.

Methods

Mosaic liver alcohol dehydrogenase (Co-Zn-ADH) was prepared as previously described (experiment 3 of Young and Wang, 1971) by dialysis of the enzyme (4 mg/ml) for 8 hr in cobaltous acetate and cobaltous chloride at pH 5.5 and 25°. Exhaustive dialysis in Tris-chloride buffer (pH 7.0 and 4°) removed extraneous metal from the active metalloenzyme. The fully substituted cobalt alcohol dehydrogenase (Co-ADH) was prepared essentially by the procedure of Santiago and Shore (1974) which is summarized as follows. (1) After dialysis at 4° against sodium phosphate buffer and then sodium sulfate buffer (pH 7.0, 0.1 *M* sulfate) the dialysis bag containing Zn-ADH (4 mg/ml) was introduced into a cobaltous sulfate solution (pH 5.5, 0.1 *M*) equilibrated under nitrogen. The apparatus used was a flask equipped with a tube entering the bottom for nitrogen flow and loosely sealed at the top. (2) The enzyme was incubated at room temperature with stirring for 4.5 days under a constant nitrogen atmosphere. Two additional changes of the cobaltous sulfate solution were made. (3) Dialysis at 4° first against Tris-chloride (pH 7.0, 0.1 *M*, 3 changes, 36 hr) and then against sodium phosphate (pH 7.0, 0.1 ionic strength, 3 changes, 36 hr) under a nitrogen atmosphere was used to free the enzyme solution from extraneous metal ions.

Alkylation of the Co-Zn-ADH and Zn-ADH was accomplished by the addition of a 50-fold excess of iodoacetamide (5 m*M*) to the nuclear magnetic resonance (NMR) sample tube containing the ternary Co-Zn-ADH-NADH-isobutyramide complex. The concentrations of the individual components were 0.12 m*M* enzyme containing 0.15 m*M* Co(II), 0.6 m*M* NADH, and 30 m*m* isobutyramide. Incubation for 36 hr in a nitrogen atmosphere at 25° resulted in 80% inactivation of the enzyme. Control samples untreated with iodoacetamide, but otherwise identical, lost 20% of their activity under these conditions.

Enzyme concentrations were determined by the method of Lowry et al. (1951) using ribonuclease as a standard. Spectroscopic measurements at 280 nm using the extinction coefficients of 0.43 mg^{-1} cm^2 for the native zinc LADH and 0.49 mg^{-1} cm^2 for Co-ADH (Drum and Vallee, 1970) established the concentration range used in the Lowry determinations. Excellent agreement between the spectroscopic and colorimetric methods was obtained for the native enzyme.

Zinc and cobalt determinations were performed with a Perkin-Elmer Model 305 atomic absorption spectrometer equipped with a Hitachi QPD33 recorder. Metal standards were used to calibrate the recorder deflection. To determine the concentration of NADH binding sites, fluorometric NADH titrations of the three enzyme systems in the presence of isobutyramide (Winer and Theorell, 1960) were accomplished on a Perkin-Elmer MPF-2A recording fluorescence spectrophotometer.

For use in Fourier transform (FT) NMR experiments, samples of the enzyme were placed in phosphate buffer in D_2O by the method described by Fung et al. (1974) using a nitrogen box in which exposure to oxygen was minimal. Sample tubes were filled and sealed inside the box before each experiment.

The longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the methyne and methyl protons of isobutyramide and the methyl and methylene protons of ethanol were measured by pulsed FT-NMR (Sloan and Mildvan, 1974) at 100 and at 220 MHz using the Varian XL-100 and HR-220 FT-NMR spectrometers equipped with nitrogen-flow temperature controls. At 100 MHz, decoupling procedures, irradiating either the resonance frequency of the methyl protons of isobutyramide or the HDO frequency, improved the signal to noise ratio for the isobutyramide methyne resonance. During the NMR experiments, some of which lasted 16 hr, no more than 20% of the enzymatic activity was lost.

Pulsed NMR measurements at 24.3 and 8 MHz of the longitudinal relaxation rate ($1/T_1$) of water protons at 23° were made on an NMR specialities PS 60W pulsed nuclear magnetic resonance spectrometer. Water proton relaxation rates (PRR) at 100 and 220 MHz at 23° were measured by the FT-NMR method, as previously described (Fung et al., 1974). Dilution effects, resulting from coenzyme and sub-

[2] Abbreviations used are: LADH, alcohol dehydrogenase from liver; Zn-ADH, native alcohol dehydrogenase from liver; Co-Zn-ADH, mosaic liver alcohol dehydrogenase containing cobalt and zinc; Co-ADH, cobalt substituted alcohol dehydrogenase from liver; IBA, isobutyramide; AcAld, acetaldehyde.

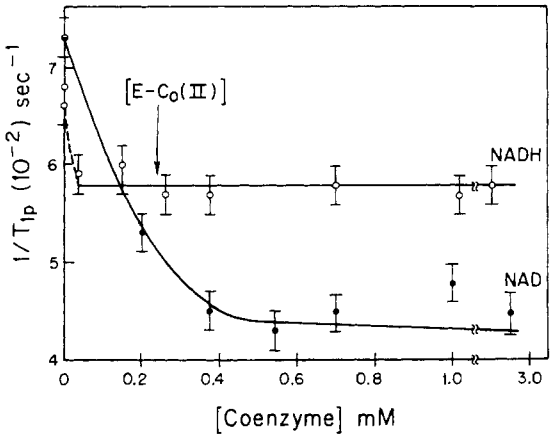

FIGURE 1: Effect of NAD^+ and NADH binding on the relaxivity of water protons in the presence of Co-Zn-ADH (0.24 mM) at 24.3 MHz and 23° in 0.1 M Tris-Cl (pH 7.0). Uncertainties in the T_1 measurements, determined by the Carr and Purcell (1954) method, are represented by error bars through the experimental points. The NAD^+ curve is computed assuming simple binding of the coenzyme to equivalent sites on Co-Zn-ADH using the K_D value of Table I.

strate additions in the titrations, were corrected for by appropriate controls (Sloan and Mildvan, 1974).

Results

Metal Ion Exchange. The exchange of Co(II) for Zn(II) accomplished after 8 hr under the conditions described above consistently produced a cobalt–zinc mosaic enzyme containing 1.28 ± 0.04 g-atoms of cobalt and 2.2 ± 0.2 g-atoms of zinc per 80,000 molecular weight. The total metal content agreed with that determined for dialyzed native liver alcohol dehydrogenase (3.8 ± 0.2 g-atoms of Zn/80,000 molecular weight). Moreover, the [Co]/[Zn] ratio, whether determined before or after D_2O exchange and the NMR experiments, remained constant.

The general rate equation for an exchange process (Friedlander and Kennedy, 1955, Frost and Pearson, 1963) simplifies to a pseudo-first-order equation with a single logarithmic term for each type of site under the present conditions in which a 10^3-fold excess of Co(II) over enzyme is present (Friedlander and Kennedy, 1955; Drum et al., 1969; Yound and Wang, 1971).

The pattern of substituting Co(II) for Zn(II) in the presence of a large (10^3-fold) excess of Co(II) over a 72-hr time range has been reported by Young and Wang (1971) using two calculated pseudo-first-order rate constants for metal exchange, 0.041 hr^{-1} for fast exchange, and 0.008 hr^{-1} for slow exchange. We have confirmed these relative rate constants, as have Takahashi and Harvey (1973). Based on these constants, 80% of the bound Co(II) is at the fast exchanging Zn(II) site and 20% is at the slowly exchanging zinc site after the 8-hr exchange. As reported by Young and Wang (1971), we found the specific activity of the mosaic enzyme (5.8 ± 0.3 units/mg) to be indistinguishable from that of the native Zn-ADH (5.8 ± 0.3 units/mg) indicating specific Co(II) substitution for Zn(II).

Exchange of up to 85% of the bound zinc for cobalt was accomplished by 4.5-day incubations in $CoSO_4$. From the metal content and the protein concentration determined by the Lowry procedure, these preparations contained 3.5 ± 0.1 g-atoms of cobalt and 0.7 ± 0.1 g-atoms of zinc per mol of enzyme. From the kinetics of metal exchange (Young and Wang, 1971, Takakashi and Harvey, 1973) essentially

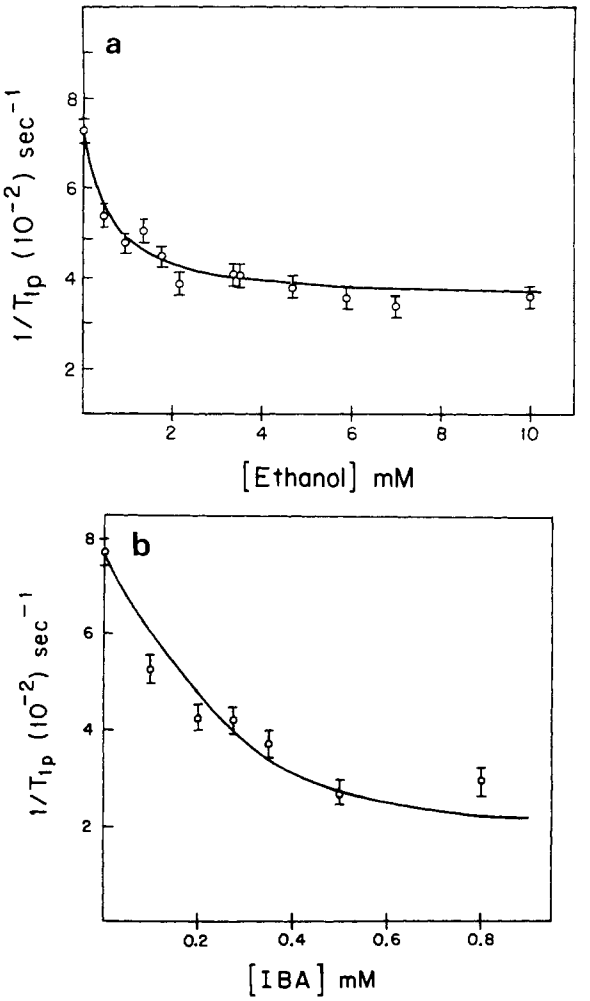

FIGURE 2: Effect of ethanol (a) and isobutyramide (b) binding on the relaxivity of water protons in the presence of Co-Zn-ADH (0.24 mM) at 24.3 MHz and 23° in 0.1 M Tris-Cl (pH 7.0). Error bars and computed curves are defined as in Figure 1.

all of the fast-exchanging metal sites should be occupied by cobalt after an exchange period of 4.5 days. As reported by Drum and Vallee (1969), and Drott et al. (1974), we found the specific activity of this preparation (4.4 ± 0.4 units/mg) to be 24% lower than that of the native enzyme. When the criterion of fluorometric titration of the enzyme with NADH in the presence of isobutyramide (Yonetani and Theorell, 1962) instead of the Lowry procedure was used to determine the LADH concentration, 10–20% higher values of 3.84 g-atoms of cobalt and 0.96 g-atom of zinc per pair of NADH binding sites were calculated suggesting that ~10% of the enzyme sites fail to bind NADH. Previously, Theorell and McKinley-McKee (1961a) found 20% lower enzyme concentrations as determined by fluorimetric titration compared with protein analyses. Extension of the incubation period resulted in further protein denaturation and little further metal ion exchange. In the magnetic resonance experiments, it has been assumed that the mosaic and fully substituted enzymes differ only in the extent of replacement of Zn(II) by Co(II).

The Binding of Coenzymes and Substrates to Co-Zn-LADH as Studied by Water Proton Relaxation Rates. At 24.3 MHz, the mosaic metal complex of liver alcohol dehydrogenase increased the longitudinal relaxation rate ($1/T_1$) of water protons by an amount significantly greater than

Table I: Comparison of Titration Parameters for Co–Zn–ADH Complexes with Kinetic and Binding Data of Zn–ADH.

Ligand	K_D (μM)	$1/T_{1p}$ (+ ligand) / $1/T_{1p}$ (− ligand)	Native (ADH) K_D (μM)	Native (ADH) K_M or K_I (μM)
Ethanol	500	0.48	4,600[b]	225–6060[e]
Ethanol (+ 6 mM NADH)	72,000	0.13	40,000–75,000[b]	130,000[f]
AcAld	10	0.56	10[b]	10–239[e]
IBA	125	0.29	930–5,750[c]	143[g]
NAD^+	10	0.60	22[d]	5.9–141[e]
NADH	1.1[a]	0.80	1.2[d]	0.28–6.7[e]

[a] From the fluorescence data of Takahashi and Harvey (1973). [b] From Theorell and McKinley-McKee (1961b). [c] From Winer and Theorell (1960). [d] From Iweibo and Weiner (1972). [e] From Theorell and McKinley-McKee (1961a). The ranges of K_M values for the substrates and for the coenzymes represent K_M from zero to infinite coenzyme and zero to infinite substrate concentrations, respectively. [f] From Shore and Theorell (1966). [g] From Theorell and McKinley-McKee (1961c).

did an equal concentration of the native zinc enzyme. The paramagnetic effect of enzyme-bound Co(II) on the longitudinal relaxation rate of water protons ($1/T_{1p}$) is given by

$$1/T_{1_p} = 1/T_1^* - 1/T_1^0 \quad (1)$$

where $1/T_1^*$ and $1/T_1^\circ$ are the relaxation rates of the paramagnetic and diamagnetic protein solutions, respectively. A decrease in $1/T_{1p}$ was observed upon titrating the mosaic enzyme with NAD^+ (Figure 1), and larger decreases were noted with ethanol (Figure 2a) and isobutyramide (Figure 2b) suggesting that NAD^+ and substrates decreased the access of water protons to the enzyme bound Co(II).

Theoretical curves based on the K_D values and relative decreases in $1/T_{1p}$ or deenhancement values which best fit the titrations are illustrated in Figures 1 and 2. A compilation of the titration parameters for ethanol, acetaldehyde, isobutyramide, and NAD^+ from several investigations is shown in Table I. Greater decreases in $1/T_{1p}$ with substrates or with the substrate analog isobutyramide than with coenzymes are detected, suggesting displacement or occlusion of water ligands by substrates on the enzyme-bound Co(II).

A very small and unexplained decrease in $1/T_{1p}$, just beyond the experimental error, was consistently observed at low concentrations of NADH (Figure 1). However, this small decrease was complete at only 15% of the equivalence point and therefore did not reflect binding to the active site of the complex. Hence, the dissociation constant for NADH from the binary complex with the mosaic enzyme was indeterminate by PRR. However, NADH binding to such a mosaic had previously been detected by Takahashi and Harvey (1973) using fluorescence enhancement. From their data we estimate a K_D value for NADH from the binary complex (1.1 μM) which agrees with that found for the native Zn enzyme (Iweibo and Weiner, 1972).

From our fluorescence titrations (Figure 3) the dissociation constant of NADH from the ternary Co-Zn-ADH-IBA-NADH complex (0.03 μM) was obtained from a Scatchard plot of the fluorescence data. Although the accuracy of the value is limited by the tightness of binding of NADH, it agrees with the value obtained for the native enzyme (0.02 μM) by Winer and Theorell (1960). A theoretical curve based on the K_D determined for the mosaic enzyme is shown in Figure 3. A linear extrapolation of the initial slope of the curve intersects the plateau at a point corresponding to 1.74 mol of NADH bound/mol of enzyme (Figure 3).

Hence the binding of NADH to the mosaic enzyme, as

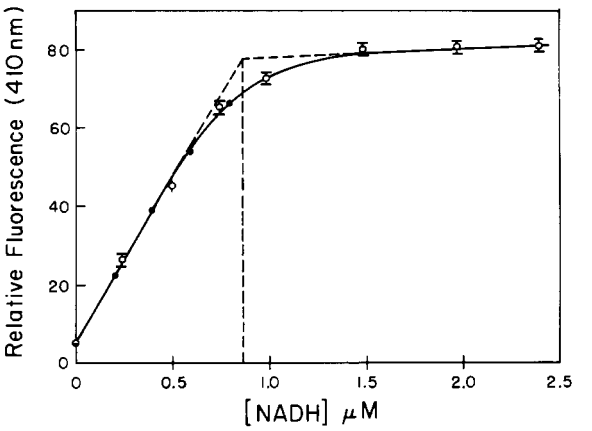

FIGURE 3: Fluorescence enhancement titration curve of the Co-Zn-ADH-IBA complex with NADH. The experiment was performed at 23° in 0.1 M Tris-Cl (pH 7.0). The enzyme concentration was 0.50 μM as determined by the Lowry et al. (1951) procedure. The solution was excited at 330 nm and emission intensity was recorded at 410 nm.

detected by fluorescence, is indistinguishable from its binding to the native enzyme, but NADH binding causes a negligible effect in $1/T_{1p}$ of water protons.

General agreement is observed between the K_D values determined by PRR and those determined by other methods (Table I). In the case of isobutyramide, the K_D determined by the $1/T_{1p}$ titration is in better agreement with the K_I value than is an early and indirect estimate of the K_D from fluorescence titrations (Table I). The possibility that the lower value of K_D for isobutyramide found in these studies might reflect the presence of residual NADH, even after exhaustive dialysis, was ruled out by fluorescence measurements. In the case of ethanol the observed K_D value also agrees better with the kinetically determined K_M value than does an early estimate of the K_D calculated from indirect fluorescence titrations (Table I). The presence of a saturating level of NADH (6 mM) increased the dissociation constant of ethanol 144-fold in accord with previous kinetic and binding data (Table I).

Water Proton Relaxation Studies of the Fully Substituted Cobalt Alcohol Dehydrogenase and Its Complexes. Increasing the Co(II) content of alcohol dehydrogenase to a value of 3.5 g-atoms/mol, a 2.5-fold increase in Co(II) content over that present in the mosaic Co-Zn enzyme, produced no increase but rather a 24% decrease in $1/T_{1p}$ at 24.3 MHz and a 19% decrease in $1/T_{1p}$ at 100 MHz for comparable enzyme concentrations (Table II). Greater decreases in $1/T_{1p}$ were observed in the substrate or coen-

Table II: Comparison of the Effects of the Mosaic (Co–Zn–ADH) and Fully Substituted (Co–ADH) Alcohol Dehydrogenases on the Relaxation Rates of Water Protons.[a,b]

	Co–Zn–ADH					Co–ADH						
	$1/T_{1p}$			$1/T_{1p}$[ADH]		$1/T_{1p}$			$1/T_{1p}$[ADH]		% Change	
	Frequency (Hz)											
Ligand	8	24.3	100	24.3	100	24.3	100	220	24.3	100	24.3	100
None	0.105	0.097	0.053	378	206	0.126	0.073	0.049	286	166	−24.3	−19.4
EtOH	0.099	0.063	0.030	245	117	0.118	0.065	0.034	268	148	(+9.4)	(+26.5)
IBA	0.082	0.052	0.026	202	101	0.064	0.022	0.026	145	50	−28.2	−50.5
NADH	0.094	0.084	0.050	327	195	0.087	0.053	0.042	198	121	−39.5	−38.0
EtOH–NADH						0.077	0.048	0.026	175	109		
IBA–NADH	0.068	0.054	0.032	210	125	0.048	0.014	0.016	109	32	−48.1	−74.4

[a] The data on the Co–Zn mosaic were obtained in solutions of 0.257 m*M* enzyme containing 1.3 Co(II)/80,000 daltons. The data on the fully substituted Co–ADH were obtained in solutions of 0.44 m*M* enzyme containing 3.5 Co(II)/80,000 daltons. [b] Errors in the $1/T_{1p}$ values at 100, 220, and 24.3 MHz are calculated to be 2.8% based on a 2% uncertainty in measuring T_1 by the null point method of Carr and Purcell (1954). At 8 MHz the uncertainty is 4% and the calculated errors in $1/T_{1p}$ are 5.7%.

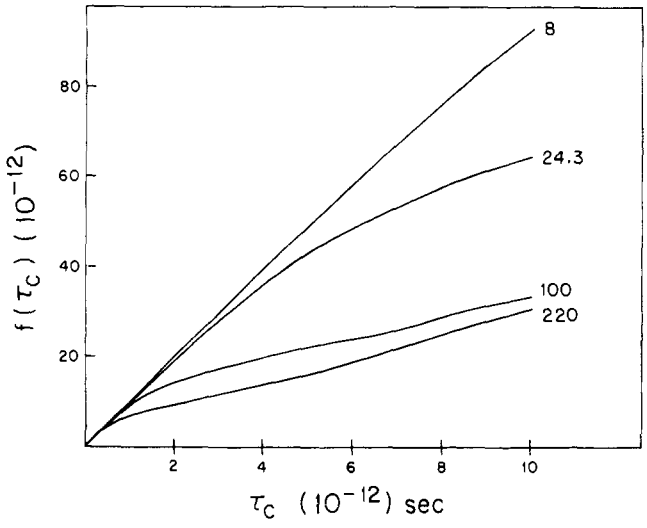

FIGURE 4: Theoretical curves for the correlation function $f(\tau_c)$, as a function of the correlation time for dipolar interaction, τ_c. Values of $f(\tau_c)$ have been calculated from eq 4 for four frequencies, 8, 24.3, 100, and 220 MHz.

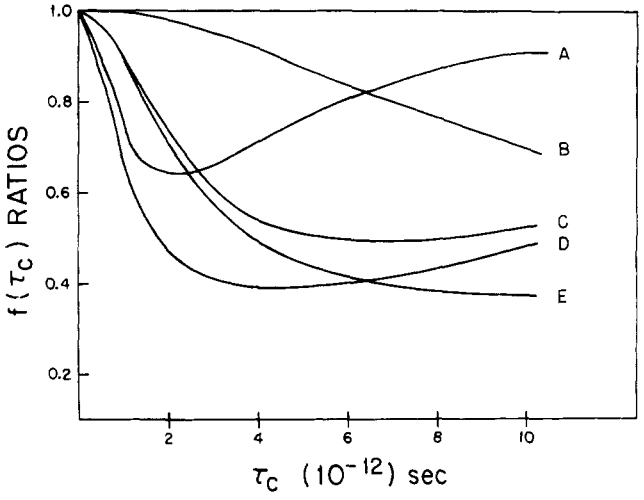

FIGURE 5: Computed curves for the frequency dependence of the correlation function $f(\tau_c)$ [expressed as $f(\tau_c)$ ratios] as a function of the correlation time τ_c. (A) 220/100 (MHz) $f(\tau_c)$ ratio; (B) 24.3/8 (MHz) $f(\tau_c)$ ratio; (C) 100/24.3 (MHz) $f(\tau_c)$ ratio; (D) 220/24.3 (MHz) $f(\tau_c)$ ratio; (E) 100/8 (MHz) $f(\tau_c)$ ratio.

zyme complexes (Table II) with the exception of the ethanol complex which showed small increases in relaxivity at 24.3 and 100 MHz. In all cases the relaxivity per bound Co(II) decreased greatly in proceeding from the Co–Zn mosaic to the fully substituted enzyme (Table II).

Although more extensive structural changes have not been ruled out, these data are most simply explained by assuming that Co(II) bound at the structural site exerts no detectable paramagnetic effect on $1/T_1$ of water suggesting that these structural sites are inaccessible to solvent. However, the replacement of Zn(II) by Co(II) at these structural sites alters the paramagnetic effect of Co(II) at the catalytic sites on $1/T_1$ of water. The frequency dependence of $1/T_{1p}$ (Table II) indicates this alteration to be due in part to a change in the correlation time at the catalytic Co(II) sites (vide infra).

Frequency Dependence of the Water Proton Relaxation Rate in Various Co(II)–Alcohol Dehydrogenase Complexes. To evaluate the correlation time τ_c for Co(II)–proton interaction, and thereby the coordination number for water (q) on the enzyme-bound Co(II), the effects of equal concentrations of Co–Zn–LADH and native LADH on the water proton longitudinal relaxation rates were determined at 8, 24.3, and 100 MHz. The paramagnetic effect ($1/T_{1p}$) normalized by the factor f = [Co(II)]/[water protons] is given by (Luz and Meiboom, 1964)

$$\frac{1}{fT_{1p}} = \frac{q}{T_{1M} + \tau_M} \qquad (2)$$

where q is the number of fast exchanging water molecules coordinated to Co(II), τ_M is the residence time, and T_{1M} the relaxation time of coordinated water ligands. The observed frequency dependence of $1/T_{1p}$ for each complex (Table II) indicates that $\tau_M \ll T_{1M}$, i.e., that values of the relaxation rates are not limited by water proton exchange. Hence $1/fT_{1p}$ is well approximated by q/T_{1M} and can be used to estimate q, the number of liganded H_2O molecules, by the expression (Solomon, 1955; Solomon and Bloembergen, 1956; Fung et al., 1974):

$$\frac{1}{fT_{1p}} = \frac{q}{T_{1M}} = q\left(\frac{C}{r}\right)^6 f(\tau_c) \qquad (3)$$

In eq 3 r is the ion-proton internuclear distance and C is a product of constants proportional to the spin state and average g value of the metal ion and numerically equal to 895

Table III: Values of the Correlation Time (τ_c) for Co(II)–Water Interaction and Coordination Number for Water Protons ($2q$) in Binary and Ternary Complexes of Co–Zn–ADH.

Complex	$\tau_c \times 10^{12}$ sec from $\frac{f(\tau_c)_{100}}{f(\tau_c)_8}$	from $\frac{f(\tau_c)_{100}}{f(\tau_c)_{24.3}}$	from $\frac{f(\tau_c)_{24.3}}{f(\tau_c)_8}$	$2q$[a]
CoZnADH–H_2O	3.8	3.9	4.0 ± 1	0.96 ± 0.20
CoZnADH–ethanol	≤8.0	4.4	≤8.2	0.53 ± 0.11
CoZnADH–IBA	≤6.3[b]	≤5.5[b]	≤7.6[b]	0.31 ± 0.16
CoZnADH–acetaldehyde				0.60 ± 0.12[c]
CoZnADH–NADH	3.5	3.3	4.5 ± 1	0.87 ± 0.20
CoZnADH–NAD^+				0.69 ± 0.14[c]
CoZnADH–NADH–IBA	4.4	3.4	≤7.2[b]	0.31 ± 0.16

[a] The q value is multiplied by 2 to give the number of fast exchanging protons rather than water molecules coordinated to the catalytic Co(II). [b] The upper limit values are based on maximized $f(\tau_c)$ ratios as deduced from the error limits in the T_1 measurements. [c] Correlation time not determined but assumed identical with the average of that of all other complexes (3.9×10^{-12} sec).

for the interaction of Co(II) with protons[3] (Fung et al., 1974). The correlation function $f(\tau_c)$

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2\tau_c^2} \quad (4)$$

is a function of the Larmor precession frequencies for nuclear (ω_I) and electron (ω_S) spins and of τ_c, the correlation time for dipolar interaction.

From eq 3 and 4 it follows that ratios of the type $f(\tau_c)_{24.3}/f(\tau_c)_{100}$ obtained directly from the ratio of the experimental values of $1/T_{1p}$ from Table II can be used to calculate τ_c. This procedure was carried out graphically using the theoretical curves shown in Figures 4 and 5 which were constructed by assuming that the frequency dependence of $1/fT_{1p}$ resulted from an ω_S rather than an ω_I dispersion (Fung et al., 1974). An ω_S dispersion results from the similarity of τ_c to $1/\omega_S$ (10^{-12} sec) rather than to $1/\omega_I$ (10^{-9} sec). The short electron spin relaxation times generally observed for complexes of yigh spin Co(II) (5×10^{-13}–10^{-11} sec) serve as τ_c (Luz and Meiboom, 1964; Fung et al., 1974). In the present case a typically short electron spin relaxation time is established by the observation that the EPR spectra of high spin Co(II) in the mosaic and fully substituted enzymes, visible at 8°K, becomes immeasurably broad at temperatures above 17°K.[4]

The $1/fT_{1p}$ values were determined from the $1/T_{1p}$ data of Table II and the titration data illustrated in Figures 1 and 2 for the Co-Zn mosaic enzyme, assuming that only the Co(II) bound at the catalytic site (i.e., 80% of the total bound Co(II)) is responsible for the paramagnetic effect on water protons. The correlation times for the various complexes of the Co-Zn mosaic enzyme (Table III) fall in the range $3.9 \pm 0.6 \times 10^{-12}$ sec. The resulting value of q for the free enzyme (Table III) indicates the presence of 0.48 ± 0.10 water ligands or 0.96 ± 0.20 water protons in the coordination sphere of the enzyme-bound Co(II) which exchange at a rate greater than $1/fT_{1p}$ or 1.7×10^4 sec^{-1}. The value of $2q$ in the Co-Zn-ADH complex of ~1 suggests a fast exchanging hydroxyl ligand on Co(II).[5] A scalar contribution to $1/fT_{1p}$ of water protons, which cannot be excluded, would decrease the value of $2q$ to below 1.

The number of fast exchanging protons in the coordination sphere of enzyme-bound Co(II) ($2q$) in the coenzyme and substrate complexes based on the titration data of Table I which are more accurate than the single values of $1/T_{1p}$ in Table II are summarized in Table III. The value of $2q$ in the NADH complex is similar to that of the free enzyme within the experimental error (Table III) suggesting that the small decrease in $1/T_{1p}$ observed at 24.3 MHz (Figure 1, Table I) may be due in part to a decrease in τ_c. A decrease in τ_c is observed between 100 and 24.3 MHz, but not between 24.3 and 8 MHz (Table III). Electron spin relaxation times often vary with frequency (Bloembergen and Morgan, 1961; Fung et al., 1974). Using the average correlation time, no significant change in $2q$ is calculated for the NAD^+ complex (Table III).

In the ethanol and isobutyramide complexes the number of fast exchanging protons ($2q$) in the coordination sphere of Co(II) is significantly decreased (Table III) and a similar effect may be operative with acetaldehyde, suggesting that substrates partially displace or hinder the exchange of the hydroxyl ligand of Co(II). The latter alternative will be shown to be correct in the ternary isobutyramide and ethanol complexes (vide infra).

Analysis of the frequency dependence of $1/T_{1p}$ of water in the presence of the fully substituted Co(II) alcohol dehydrogenase reveals small but significant decreases in τ_c at the catalytic Co(II) site (≤22%) for all complexes when Co(II) replaces Zn(II) at the structural site. The correlation times for the various complexes fall in the range $2.9 \pm 0.7 \times 10^{-12}$ sec.[6] The resulting values of q (again based on the concentration of Co(II) at the catalytic sites) agree with those determined for the mosaic enzyme complexes (Table III) within the experimental error of this parameter.

Effects of Co-Zn-ADH on the Relaxation Rates of the Methyl and Methyne Protons of Isobutyramide. Table IV shows the results of several determinations of the paramagnetic effect of the mosaic enzyme on the relaxation rates of

[3] The mean value of $C = 895$ is calculated from the average g values of various tetrahedral and pentacoordinate Co(II) complexes ($C = 885 \pm 30$; Kennedy et al., 1972) and from preliminary EPR measurements on the mosaic and fully substituted enzymes ($C = 905 \pm 10$; Vallee et al., 1974, and H. Drott, private communication).

[4] E. Melamud, D. L. Sloan, and J. M. Young, unpublished observations.

[5] The relaxivity experiments generally detect the ligand which escapes from the enzyme. In the present case, this ligand appears to be a hydroxyl ion. When on the enzyme, this ligand could be either a hydroxyl ion or a hydrogen bonded water molecule. In the latter case the water ligand would leave a proton behind on the enzyme when it dissociates.

Table IV: Longitudinal and Transverse Relaxation Rates of Isobutyramide Protons in the Presence of the Co–Zn–ADH.[a]

Expt	Proton	100 MHz, $(sec^{-1}) \times 10^2$		220 MHz, $(sec^{-1}) \times 10^2$		r (Å)[b]
		$1/T_{1p}$	$1/T_{2p}$	$1/T_{1p}$	$1/T_{2p}$	
I, CoZnADH–IBA[c]	$-CH_3$	5.4 ± 0.9	55 ± 16	3.9 ± 0.7	83 ± 17	9.1 ± 0.3
II, CoZnADH–IBA–NADH	$-CH_3$	25 ± 4.4	101 ± 29	18 ± 3.2	126 ± 25[d]	7.0 ± 0.2
(O_2 atm)	–CH–	21 ± 6.3	253 ± 36			7.2 ± 0.3
III, CoZnADH–IBA–NADH	$-CH_3$	21 ± 3.5	216 ± 31			7.2 ± 0.2
(N_2 atm)	–CH–	35 ± 10	314 ± 88			6.6 ± 0.3
IV, CoZnADH–IBA–NADH	$-CH_3$	18 ± 2.9	63 ± 18			7.4 ± 0.2
(N_2 atm) + iodoacetamide	–CH–	30 ± 7.9	75 ± 21			6.8 ± 0.3

[a] Concentrations were 30 m*M* IBA, 0.12 m*M* CoZnADH (0.15 m*M* cobalt), and 0.6 m*M* NADH. $1/f$ equaled 199. [b] A τ_c value of 1.3×10^{-12}, based on the ratio of $1/fT_{1p}$ at 100 and 220 MHz for protons of isobutyramide, was used in the distance calculations. [c] When experiments I, II, and III were repeated the values of $1/T_{1p}$ and $1/T_{2p}$ were found to be reproducible within the error limits of the T_1 and T_2 measurements. Experiment IV was not reexamined. [d] Based on the width of the signal at half-height.

Table V: Longitudinal and Transverse Relaxation Rates of Isobutyramide Protons in the Presence of Fully Substituted Co(II) Alcohol Dehydrogenase under Nitrogen Atmosphere at 23°.[a]

Complex	Proton	100 MHz		220 MHz		r (Å)[b]
		$1/T_{1p}$ $(sec^{-1}) \times 10^2$	$1/T_{2p}$ $(sec^{-1}) \times 10^2$	$1/T_{1p}$ $(sec^{-1}) \times 10^2$	$1/T_{2p}$ $(sec^{-1}) \times 10^2$	
CoADH–IBA + NADH	$-CH_3$	22 ± 3.3[c]	165 ± 23	17 ± 2.5	59 ± 12	7.6 ± 0.2
	–CH–	29 ± 7.4	413 ± 116	25 ± 12[d]	66 ± 30	7.2 ± 0.3

[a] Concentrations were 30 m*M* IBA and 0.075 m*M* CoADH (0.25 m*M* Co(II)), and 0.6 m*M* NADH. $1/f$ equaled 121. [b] A τ_c value of 1.0×10^{-12} based on the ratio of $1/fT_{1p}$ at 100 and 220 MHz of the methyl protons of IBA was used in the distance calculations. [c] Measurements of the paramagnetic effect of Co(II) on $1/T_1$ and $1/T_2$ were repeated and found to be reproducible within the error of the T_1 and T_2 measurements under anerobic conditions. The effect was reduced considerably in the presence of oxygen. [d] Methyne proton relaxation rates at 220 MHz were measured on the couplet septet.

the six methyl protons and the single methyne proton of isobutyramide. The stability of Co–Zn–ADH in an oxygen atmosphere has been documented (Young and Wang, 1971) and no special precautions were taken to eliminate O_2 in most of the NMR experiments. However, as a basis for comparison with studies involving the fully substituted cobalt enzyme, which is sensitive to oxidation, measurements of $1/fT_{1p}$ and $1/fT_{2p}$ of the Co–Zn–ADH–IBA complex were made under a N_2 atmosphere as well. The results were indistinguishable within experimental error (Table IV). The longitudinal relaxation rates of the isobutyramide methyne proton measured with or without decoupling of the methyl protons as described in the Methods section were in good agreement. Because of the high affinity of alcohol dehydrogenase for isobutyramide in all of the complexes studied (Table I) no corrections of the relaxation rates for site occupancy were required. Values of r for the methyl protons (Table V), calculated from $1/fT_{1p}$ values, indicate that isobutyramide binds at a site far removed from the catalytic metal.[6] Ternary complex formation with NADH shifts the bound inhibitor toward the cobalt ion. In all cases $1/fT_{2p}$ is significantly greater than $1/fT_{1p}$, and both relaxation rates show a frequency dependence. By these criteria the observed $1/fT_{1p}$ and $1/fT_{2p}$ values are not limited by chemical exchange (Nowak and Mildvan, 1972; Fung et al., 1974). Thus the absolute distances calculated for both types of protons from $1/fT_{1p}$ in this system are limited only by the errors in the measurements of $1/fT_{1p}$ and by the success in separating the paramagnetic contributions of the two types of bound paramagnetic metal atoms.

Comparison of the Effects of Fully Substituted and Mosaic Dehydrogenases on the Relaxation Rates of the Methyl and Methyne Protons of Isobutyramide. The observed relaxation rates of the methyl and methyne protons of isobutyramide complexed with the fully substituted Co–ADH and NADH are compiled in Table V. The most interesting aspect of the results is that the average r values from all Co(II) ions are slightly larger than those determined for the mosaic enzyme. The differences do not result from a change in the correlation time, since $f(\tau_c)$ values for the Co–ADH enzyme were determined to be smaller than those determined for Co–Zn–ADH (Tables V and VI) and this would shorten rather than lengthen the apparent distances.[6] The most likely interpretation would be that the observed $1/fT_{1p}$ reflects the sum of two paramagnetic effects: (1) a paramagnetic contribution from the catalytic Co(II) atoms at a distance approximately 7 Å from the substrate protons; and (2) a contribution from the structural Co(II) atoms far removed from protons. This suggestion is substantiated in the following way.

From the kinetics of cobalt exchange discussed above, and from atomic absorption measurements the concentrations of cobalt (in g-atoms per 80,000 molecular weight) at the catalytic and structural sites were calculated to be 1.05 and 0.25, respectively, for the Co(II)–Zn(II) mosaic ADH. Similarly concentrations of 2.0 and 1.5 g-atoms per 80,000

[6] Within the range of our measured values of τ_c (Tables III–VI) no consistent increase in τ_c with frequency was detected indicating no systematic frequency dependence of τ_c. Hence, distances were calculated using the average correlation time calculated over the frequency range 100–220 MHz. This assumption introduces an uncertainty well below 10% in the distance calculations except in the case of the ethanol complex where the uncertainty is somewhat larger (see Table VI).

Table VI: Longitudinal and Transverse Relaxation Rates of Ethanol Protons in the Presence of Co–Zn–ADH at 100 MHz and 23°.[a]

Experiment	Proton	$1/T_{1p}$ (sec^{-1}) × 10^2	$1/T_{2p}$ (sec^{-1}) × 10^2	$1/T_{1M}$[b] (sec^{-1}) × 10^2	r (Å)
I, CoZnADH	$-CH_3$	3.9 ± 0.8	28 ± 3.2	1.4 ± 0.3	13.9 ± 0.7[c]
– ethanol	$-CH_2-$	9.0 ± 0.8	32 ± 3.2	3.3 ± 0.3	12.0 ± 0.6[c]
II, CoZnADH	$-CH_3$	17 ± 8.1[d]	22 ± 3.2	103 ± 48	5.5 ± 0.6[e]
– ethanol + NADH					6.8 ± 0.6[c]
	$-CH_2-$	15 ± 8.5	39 ± 12	89 ± 50	5.7 ± 0.7[e]
					6.9 ± 0.7[c]

[a] Concentrations were 4 m*M* ethanol, 0.12 m*M* CoZnADH (0.15 m*M* cobalt), and 0.6 m*M* NADH. $1/f$ equaled 24.7. [b] From $1/fT_{1p}$, correcting for occupancy of the enzyme sites from the K_D values for ethanol (Table I) and for the presence of 80% of the bound Co(II) at the catalytic site as discussed in the text. [c] The value of $\tau_c = 4.4 \times 10^{-12}$ sec as determined by frequency dependence studies of $1/T_{1p}$ of water protons in the CoZnADH–ethanol complex (Table II) was used for these distance calculations. [d] Experiment I was repeated and the values of $1/T_{1p}$ and $1/T_{2p}$ were reproducible within the error limits of the T_1 and T_2 measurements. Experiment II was carried out twice with two independent enzyme preparations with equivalent activities and metal content. The variabilities of the $1/T_{1p}$ and $1/T_{2p}$ values, which were outside the error limits of the T_1 and T_2 measurements, were used as error limits in the distance calculations. [e] The value of $\tau_c = 0.6 \pm 0.3 \times 10^{-12}$ sec as determined by the frequency dependence of $1/T_{1p}$ of the methyl and methylene protons of ethanol (at 100 and 220 MHz) was used for these distance calculations.

molecular weight, respectively, were calculated for the fully substituted Co(II) enzyme. Distance estimates between the protons of isobutyramide and the catalytic (r_c) and structural (r_s) metal sites were made using the simultaneous

$$\frac{1.3}{(r_1)^6} = \frac{0.25}{(r_s)^6} + \frac{1.05}{(r_c)^6} \quad (5)$$

$$\frac{3.5}{(r_2)^6} = \frac{1.5}{(r_s)^6} + \frac{2.0}{(r_c)^6} \quad (6)$$

equations (eq 5 and 6) which take into account the correlation times of 1.3×10^{-12} and 1.0×10^{-12} sec determined for the mosaic and fully substituted enzymes, respectively, between 100 and 220 MHz.[6] In these equations, r_1 and r_2 represent experimentally determined distances for the Co–Zn–ADH and Co–ADH systems shown in Tables IV and V. For the methyl protons of isobutyramide r_c equals 6.9 ± 0.1 Å and r_s equals 11.4 ± 0.2 Å. For the methyne proton r_c equals 6.6 ± 0.1 Å when the average distance from Table IV is employed in the calculations. By the same criteria r_s equals 8.6 ± 1.0 Å. Clearly the catalytic site is responsible for the major portion of the observed paramagnetic effects on the proton relaxation rates of isobutyramide.

Effects of Co–Zn–ADH on the Relaxation Rates of the Methyl and Methylene Protons of Ethanol. The normalized longitudinal ($1/fT_{1p}$) and transverse ($1/fT_{2p}$) relaxation rates of the methyl and methylene protons of ethanol in the presence of Co–Zn–ADH were measured at 100 MHz and 23° (Table VI). In the binary complex of Co–Zn–ADH with ethanol, $1/fT_{1p} < 1/fT_{2p}$ indicating that the former relaxation rate is not limited by chemical exchange. As pointed out in detail elsewhere (Nowak and Mildvan, 1972) $1/fT_{1p}$ may therefore be used to calculate distances (r) between protons undergoing relaxation and the bound metal using eq 3. The value of T_{1M}, the relaxation time of the protons of bound substrate, is given by fT_{1p} in the limit of fast exchange (Luz and Meiboom, 1964). In calculating $1/T_{1M}$, a small correction to $1/fT_{1p}$ was made from the K_D of ethanol (Table I) to account for the incomplete occupancy (88.9%) of the enzyme sites by ethanol under these conditions. The τ_c value of 4.4×10^{-12} sec determined from the frequency dependence of $1/T_{1p}$ of water protons in the same complex (Table II) was used to calculate the first set of distances given in Table VI.[6] As shown in Table VI the transverse relaxation rates of ethanol were unaffected by addition of NADH but $1/fT_{1p}$ for the methyl group increased. The values of $1/fT_{2p}$ for both the methyl and methylene protons in this complex decreased with increasing temperature between 4 and 29° with large activation energies of 9.0 ± 3.2 kcal/mol and 18.8 ± 4.2 kcal, respectively, indicating that $1/fT_{2p}$ is not exchange limited.[7] Hence $1/fT_{1p}$ cannot be exchange limited and may also be used for distance calculations (Mildvan and Cohn, 1970).

The binding of NADH to Zn–ADH is known to increase the dissociation constant of ethanol by two orders of magnitude to values of 40–140 m*M* (Table I). Similarly with Co–Zn–ADH, a high dissociation constant of 72 ± 20 m*M* was obtained by titration with ethanol measuring $1/T_{1p}$ of water protons (Table I). A simple hyperbolic titration curve was observed up to 240 m*M* ethanol and no deviation from hyperbolic behavior was noted at 1 *M* ethanol which argues against denaturation by ethanol.[7] Moreover, Brooks et al, (1972) observed no discontinuous change in kinetic properties of LADH at 1 *M* ethanol. The relaxation rates of ethanol in the abortive enzyme–NADH–ethanol complex must be corrected for the low occupancy of enzyme sites by ethanol (5.25%) under the conditions of the NMR experiment. The resulting values of $1/T_{1M}$ for the methyl and methylene protons of ethanol in the abortive ternary complex together with τ_c determined by the frequency dependence of $1/T_{1p}$ of the protons of ethanol or water yield the distances shown in Table VI. As with isobutyramide, the large decreases in the distances from Co(II) to the protons of ethanol as NADH binds to the enzyme indicate a protein conformation change upon ternary complex formation.

Discussion

Proximity of two of the four zinc ions to the catalytic center of liver alcohol dehydrogenase has been suggested by a combination of X-ray (Branden et al., 1973), nuclear relaxation (Sloan and Mildvan, 1974), and electron spin resonance data on the mosaic enzyme (Drott et al., 1974). The present studies of the paramagnetic effect of Co(II) substituted alcohol dehydrogenases on the relaxation rates of water protons are interpreted most simply as indicating that

[7] The high and unequal activation energies for $1/fT_{2p}$ of the methyl and methylene protons of ethanol are atypical for the temperature dependence of the electron spin relaxation time (Mildvan and Cohn, 1970, Fung et al., 1974) and suggest a structural change in the ternary complex with temperature.

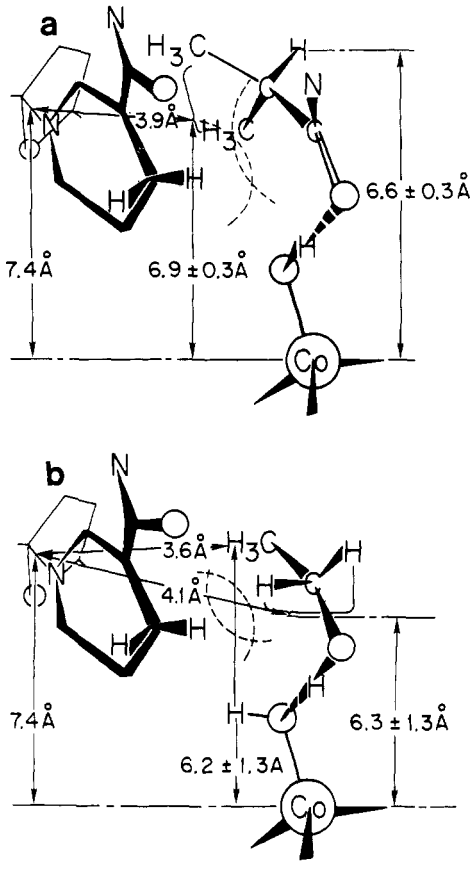

FIGURE 6: Geometry in solution of the interaction of isobutyramide (a) and ethanol (b) with cobalt and NADH at the catalytic site of alcohol dehydrogenase from liver. The distances from Co(II) to isobutyramide and to ethanol are from the present work. The distances from isobutyramide and from ethanol to NADH were estimated by Mildvan and Weiner (1969) and that from Co(II) to NADH by Drott et al. (1974). The dashed arcs represent the van der Waals radii of the atoms which are in contact.

Co(II) at the rapidly exchanging site is exposed to the solvent, while Co(II) bound at the slowly exchanging site has no measurable paramagnetic effect on water protons and presumably receives ligands only from the protein. Thus, the magnetic resonance data suggest that the more easily exchangeable Zn(II) sites are accessible to the solvent and correspond to those at the active site detected by X-ray (Branden et al., 1973) and by spectroscopic methods (Drum and Vallee, 1969b).

The number of fast exchanging water protons ($2q$) in the coordination sphere of enzyme bound Co(II) of the mosaic Co-Zn-ADH is calculated to be 0.96 ± 0.20 (Table III) and suggests a fast exchanging hydroxyl ligand on Co(II).[5] The assumption has been made that only the Co(II) bound at the catalytic site (i.e., 80% of the total bound Co(II)) is responsible for the paramagnetic effect on water protons. This assumption is justified in the following way. Replacement of Zn(II) by Co(II) at the buried sites *decreases* the paramagnetic effects of the exposed Co(II) sites on the protons of water (Table II). Thus, replacement of Zn(II) by Co(II) at the structural sites alters the local environment of the metal ion at the catalytic site resulting in a conformation change in the totally Co(II) substituted alcohol dehydrogenase. Such a conformation change may be one factor contributing to the 20% decrease in enzymatic activity generally observed with the totally cobalt (II) substituted enzyme (Drum and Vallee, 1970; Drott et al., 1974).

The binding of the coenzymes NADH or NAD^+ decreased the paramagnetic effects of Co(II) bound at the catalytic site on $1/fT_{1p}$ of water protons by 20 and 40%, respectively (Table II). These small decreases may result in part from changes in the correlation time and in the number of fast exchanging protons although the large error in $2q$ renders this point uncertain (Table III). Shore et al. (1974) have shown that saturation with NAD^+ causes the release of 0.5 proton per active site which is within our experimental uncertainty for the change in water protons near the metal ion (Table III). It should be noted that the proton detected by Shore et al. (1974) may not be near the metal ion but may be coupled to it via a charge relay system (Eklund et al., 1974).

The binding of the substrates ethanol and acetaldehyde decreased the paramagnetic effects of Co(II) bound at the catalytic site on $1/fT_{1p}$ of water protons by 52 and 44%, respectively (Table II). Unlike the effects of the coenzymes, the frequency dependence of $1/fT_{1p}$ of the ethanol complex indicates that the decrease in relaxivity is due to a significant decrease in q (Table III). In the presence of the substrate analog isobutyramide, which binds to the mosaic enzyme with an affinity consistent with its K_I (Table I), still greater decreases in q were observed. Thus substrates and substrate analogs either replace or occlude the exchange of the hydroxyl ion coordinated to Co(II) at the catalytic center. In the case of isobutyramide, and ethanol, the latter alternative has been shown to be true (discussed below).

In the case of the substrate analog isobutyramide, a distance of 9.1 ± 0.3 Å has been calculated between the catalytic Co(II) ion and the CH_3- protons of isobutyramide in the binary complex formed with the mosaic enzyme. Upon addition of NADH this distance decreased by 2.2 Å to 6.9 ± 0.2 Å, and that between the Co(II) ion and the -CH- proton was calculated to be 6.6 ± 0.3 Å, indicating a conformation change in the protein. In the ternary complex with isobutyramide and NADH, the absolute distances are too great by 2.8 ± 0.2 Å for direct coordination of the carbonyl oxygen or amide nitrogen to the catalytic Co(II) ion, but are appropriate for the formation of a second sphere isobutyramide complex (Figure 6a).[8]

Similar effects were observed in the complexes of ethanol. The calculated distances from Co(II) at the catalytic site to the $-CH_3$ and $-CH_2-$ protons of ethanol were 13.9 ± 0.7 and 12.0 ± 0.6 Å, respectively. These values exceed by ≥8.5 Å those required for direct coordination of ethanol by the cobalt ion. In the abortive ternary complex (Co-Zn-ADH-ethanol-NADH), the distances from Co(II) to the methyl and methylene protons of ethanol (Table VI) indicate that binding of NADH caused the Co(II) to $-CH_3$ distance to decrease by at least 5.8 Å and the Co(II) to $-CH_2-$ distance to decrease by at least 3.8 Å. These results establish a protein conformation change upon ternary complex formation which effects an apparent shift of the substrate toward the metal atom at the catalytic site.

The average distances from Co(II) to the methyl (6.2 ± 1.3 Å) and methylene protons (6.3 ± 1.3 Å) (Table VI) are too great by 1.7 ± 1.3 and 3.2 ± 1. 3 Å, respectively, for direct coordination of the hydroxyl group of ethanol. The lat-

[8] Lower limit distances from the catalytic Co(II) to the methyl (>6.6 Å) and methyne protons (>6.3 Å) of isobutyramide in the ternary complex, calculated by making the extreme assumption of no paramagnetic effects due to the structural Co(II) are also well beyond the values required for direct coordination (4.2 and 4.0 Å, respectively).

ter distance provides a more critical test for the lack of direct coordination. Both distances are appropriate for a second sphere complex in which a coordinated water or hydroxyl group intervenes between the metal and the substrate (Figure 6b).

Iodoacetamide is known to bind specifically to cysteine-43 of yeast alcohol dehydrogenase. Cysteine-46, the corresponding essential sulfhydryl residue on the liver enzyme, has been proposed by Drum et al. (1969a) as a possible ligand for the catalytic zinc. Alkylation of the NADH–isobutyramide–mosaic enzyme complex with iodoacetamide resulted in negligible increases ($\leq$0.2 Å) in the cobalt–isobutyramide distances (Table IV). Thus, alkylation of essential sulfhydryl groups produces little or no change in the position of cobalt at the catalytic site.

From the Co(II)–isobutyramide and Co(II)–ethanol distances reported here and other distances estimated in solution on liver alcohol dehydrogenase (Table VI) a model for the active site geometry of LADH was constructed (Figure 6). This model places the carbonyl carbon of isobutyramide in molecular contact with the C_4 hydrogen projecting from the A-face of the dihydropyridine ring (Figure 6a), consistent with the stereospecificity of hydride transfer catalyzed by alcohol dehydrogenase (see Levy et al., 1962). Figure 6 illustrates the proposed second sphere complexes of isobutyramide and ethanol in which a hydroxyl ion (or water molecule) remains coordinated to the catalytic Co(II), but its exchange is slowed to a value less than 10^4 sec^{-1} in the presence of the substrate or analog.[5]

From distances determined in solution on spin-labeled yeast alcohol dehydrogenase (Sloan and Mildvan, 1974) a model for the total conformation of coenzyme and isobutyramide at the active site was obtained. This conformation in solution agreed well with that of ADP-ribose relative to Zn(II) in the crystalline liver enzyme observed by X-ray diffraction at 2.9-Å (Branden et al., 1973) and 2.4-Å resolution (Eklund et al., 1974).

Lacking X-ray data on the pyridine–ribose conformation and substrate position on liver alcohol dehydrogenase, Eklund et al. (1974) have used such data from lactate dehydrogenase (Chandrasekhar et al., 1973) to propose an inner sphere Zn(II) alcoholate complex on liver alcohol dehydrogenase, although the Zn(II) to substrate distances from such model building are too imprecise to rule out a second sphere complex (M.G. Rossman, private communication). This extrapolation from one dehydrogenase to another is probably not valid as far as the catalytic site is concerned (Blake, 1974) because of the known differences in pyridine–ribose conformations among various dehydrogenases (Sloan and Mildvan, 1974, Rossman et al., 1974) and because lactate dehydrogenase is not a metalloenzyme.

The geometry of the active site complex for the cobalt substituted liver alcohol dehydrogenase (Figure 6) can be used to propose a mechanistic role for the metal (Figure 7), assuming the substrate acetaldehyde and its competitive inhibitor, isobutyramide, to be identically positioned (Theorell and McKinley-McKee, 1961c). The cobalt ion does not interact directly with the substrate but activates a coordinated water ligand (or hydroxyl ion) which polarizes the acetaldehyde carbonyl oxygen, through hydrogen bonding. Carbonyl polarization would facilitate hydride transfer and is consistent with recent substituent (Klinman, 1972; Jacobs et al., 1974) and kinetic isotope effects with alcohol dehydrogenase (Klinman, 1972). Subsequent protonation of the carbonyl oxygen, by either the coordinated water (Figure 7) or a protein side chain to form ethanol, would complete the reaction sequence. The group labeled B in Figure 7 may correspond to serine-48 which has been found to be near the catalytic Zn(II) (Eklund et al., 1974).

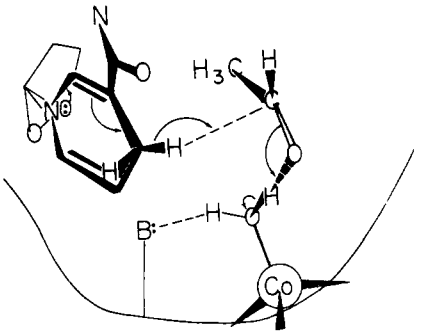

FIGURE 7: Proposed mechanism of alcohol dehydrogenase based on the geometry of the active site in solution.

A second sphere enzyme–metal–ligand–substrate complex was first detected in solution with enolase (Nowak et al., 1973). Second sphere complexes have since been detected with five enzymes which, like alcohol dehydrogenase, polarize the carbonyl groups of their substrates, namely pyruvate carboxylase (Fung et al., 1973), pyruvate kinase (Fung et al., 1973), transcarboxylase (Fung et al., 1974), ribulose diphosphate carboxylase (Miziorko and Mildvan, 1974), and malic enzyme.[9] Mechanisms analogous to that proposed here may be operative with these enzymes.

Finally, the role of the structural metal ion in liver alcohol dehydrogenase has not been directly determined. Our estimates of the distances from the structural Co(II) to the methyl protons (11.4 $\pm$ 0.2 Å) and the methyne proton (8.6 $\pm$ 1.0 Å) of isobutyramide in the ternary complex in solution confirm the observation of Branden et al. (1973) that the structural metal is far removed from the catalytic site. However, replacement of Zn(II) with the 0.16 Å smaller Co(II) ion at the structural site changes the ligand conformation at the catalytic Co(II) as reflected by a change in water relaxation (Table II), although more extensive structural changes have not been ruled out. Similar changes in τ_s may occur on binding of NADH (Table III) which is known to cause conformation changes (Tables IV and VI). Hence, a role of the structural zinc in the native enzyme may be to ensure the correct active site conformation.

Acknowledgment

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