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Metal Ion Substitution at the Catalytic Site of Horse-Liver Alcohol Dehydrogenase: Results from Solvent Magnetic Relaxation Studies. 1. Copper(II) and Cobalt(II) Ions†

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ABSTRACT: The influence of paramagnetic Cu^{2+} and Co^{2+} ions, substituted for Zn^{2+} ions at the catalytic sites of native alcohol dehydrogenase from horse liver (EC 1.1.1.1), on the nuclear magnetic spin–lattice relaxation rates of solvent water and substrate (CH_3OD) protons was studied as a function of magnetic field strength. For the Cu^{2+} ions, the data can best be fit to a model in which the resulting "blue copper" center (type I) of the enzyme is characterized by inner sphere coordinated water or substrate, both more strongly bound in the binary complex of protein with coenzyme and displaced from the ternary complex with pyrazole. In the binary complex with pyrazole, a pentacoordinated species is indicated; thus, the coordination number is reduced upon formation of the enzyme–pyrazole–coenzyme ternary complex. Although the Cu^{2+}–enzyme is able to bind coenzyme, thereby distorting its metal-binding site, it cannot discriminate significantly between alcohol substrates and water. The resulting relatively weak binding of alcohol is sufficient to explain the observed absence of enzymatic activity of the Cu^{2+}-substituted protein under the usual experimental conditions. This is the first example of a blue copper protein for which the Cu^{2+} ion is accessible to solvent. The correlation times for the paramagnetic dipolar interaction between the solvent protons and the Cu^{2+} ion are unusually short, presumably due to a strong spin–orbit interaction of the electronic spins of the Cu^{2+} ions with their thiol-sulfur ligands. The magnetic spin–lattice relaxation rates of both solvent water protons and solvent methanol methyl protons were also measured for solutions of the native enzyme, the enzyme with Zn^{2+} ions removed from the catalytic sites, and with Co^{2+} ions specifically substituted for Zn^{2+} ions at the catalytic sites. We could detect no paramagnetic contribution from the Co^{2+} ions to the magnetic relaxation rate of the solvent water and methanol protons, despite attempts to enhance the detection of paramagnetic effects by altering a variety of experimental parameters, including temperature and ionic content of the solvent, and by the addition of coenzyme and inhibitors. There are small differences in the diamagnetic contributions to the relaxation rates of the native, demetalized, and Co^{2+}-substituted enzymes that change sign with magnetic field; these small variations can readily be mistaken for true paramagnetic effects when analysis of the relaxation data is limited to the high values of magnetic field strength usually used for measurements of relaxation enhancement. As a result, previous high-field data require reinterpretation: any paramagnetic effects that may be present are small and not easily separable from a variety of small diamagnetic effects that depend on solvent composition. The reason seems to be an unusually short correlation time for the interaction between solvent protons and the Co^{2+} ions, due to a strong spin–orbit interaction of the electronic spins of the Co^{2+} ions, as was found for the Cu^{2+}-substituted enzyme.

Native horse-liver alcohol dehydrogenase (EC 1.1.1.1; ADH),[1] a dimeric enzyme of 80000 daltons, contains two Zn^{2+} ions per identical monomeric subunit, one ion at the catalytic site and a second ion at a site ~20 Å from the first [for reviews, cf. Brändén et al. (1975) and Brändén & Eklund (1978)]. Recently, techniques have been developed by which various divalent ions can be substituted specifically for the Zn^{2+}

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[1] Abbreviations used: ADH, horse-liver alcohol dehydrogenase; NMRD, nuclear magnetic relaxation dispersion; NADH and NAD^+, respectively, reduced and oxidized nicotinamide adenine dinucleotide; A(c)B(n)-ADH, enzyme with divalent metal ions of type A in the catalytic sites and divalent metal ions of type B in the noncatalytic sites; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.

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ions at the catalytic sites, leaving the occupation of the other sites unaltered (Maret et al., 1979). It is found that substitution of Co^{2+} ions yields a hybrid enzyme that is active (Maret et al., 1979), whereas substitution of Cu^{2+} ions yields a complex that binds coenzyme but lacks catalytic activity (Maret et al., 1980).

Both Co^{2+} and Cu^{2+} ions are paramagnetic when bound at the catalytic site, as evidenced by the existence of characteristic electron spin resonance signals at low temperatures (Maret et al., 1980, and unpublished results). Because of this and because alcohol as substrate presumably competes with solvent water for at least one binding site at the catalytic metal ion, we have chosen to investigate the binding of various metal ions to the protein, as well as the access of both substrate and solvent to the protein-bound ions, by measuring the influence of ion–protein complexes on the spin–lattice magnetic relaxation rate of solvent water protons and solvent methanol methyl protons as a function of magnetic field strength. This technique, called NMRD,[1] is now highly refined and can be used to measure the kinetics of metal binding to protein [cf. Brown et al. (1977)] as well as the interaction of solvent molecules with paramagnetic centers on protein molecules [for a recent critical survey of a related, but more limited, approach, cf. Burton et al. (1979)]. Additionally, NMRD measurements can be used, under certain conditions, to study the binding of nonmagnetic ions to protein by following their competition with paramagnetic ions (Koenig et al., 1978a).

We have investigated the interactions of water and methanol with Cu^{2+}- and Co^{2+}-substituted ADH, as well as the interaction of Zn^{2+}, Co^{2+}, Cd^{2+}, and Mn^{2+}, with the enzyme demetalized at the catalytic site. We have divided the reporting of the results into two papers: this paper deals with interactions of solvent and substrate with Cu(c)Zn(n)-ADH[1] and Co(c)Zn(n)-ADH under equilibrium conditions; the following paper (Andersson et al., 1981) deals with studies of the kinetics and stoichiometry of binding of Mn^{2+}, Zn^{2+}, and Cd^{2+} to the demetalized enzyme.

Experimental Procedures

Materials. Enzyme and coenzymes were obtained from Boehringer, Mannheim; pyrazole was from Ega-chemie (Steinheim); *tert*-butyl alcohol was from Aldrich, Milwaukee, WI, and distilled before use; CH_3OD was from Merck Sharp & Dohme, Canada. Preparation of Cu^{2+}-substituted protein by metal exchange in suspensions of enzyme crystals, as well as assays of the preparations, was as previously described (Maret et al., 1979, 1980). Enzyme solutions were prepared by dissolving sufficient crystalline material in buffer (generally 0.025 M Tes, pH 6.9) containing 0.2–0.3 M KCl or NaCl to obtain enzyme concentrations in the range 1.5–2 mM in monomeric units[2] (~10% by weight). Coenzyme, inhibitor, and substrate were added by microsyringe from concentrated stock solutions to minimize dilution of protein. For samples in D_2O solution, the crystals were first dialyzed extensively against D_2O with added *tert*-butyl alcohol (to keep the material crystalline and thereby prevent reduction of the Cu^{2+} ions), washed with D_2O, and otherwise treated analogously to the other preparations. Further details for specific samples are given along with the data.

Methods. NMRD measurements of methanol methyl and water protons in the solvent were made by procedures previously described [cf. Brown et al. (1977) and Hallenga & Koenig (1976)]. The method is akin to magnetic resonance experiments, and the physical quantity measured is the decay of the total magnetization of all the solvent protons. In every case, the relaxation rate of the protons is obtained by a least-squares fit to an exponential decay of at least 15 measured values of magnetization at as many different values of time. The root mean square deviation of the data from the computed exponential is generally less than ±0.5%, indicating both that the experimental scatter is small and that the time dependence of the magnetization is well represented by an exponential decay. This latter point becomes relevant to some NMRD data for protons of CH_3OD in deuterated solutions (see below) because of possible contributions to the signal from residual solvent water protons that, in principle, could relax at a rate different from that of the methyl protons. The reproducibility of the data from sample to sample, usually limited by the reproducibility of the sample composition, was generally within ±1%. Unless otherwise indicated, all data were taken at 5 ± 1 °C.

For the Cu^{2+}-substituted enzyme, paramagnetic effects predominate. The paramagnetic contribution to the relaxation rate, $1/T_{1p}$, is fit to the simplified Solomon–Bloembergen–Morgan expressions [cf. Koenig (1978)]

$$\frac{1}{T_{1p}} = \frac{f}{T_{1M} + \tau_M} \quad (1)$$

$$\frac{1}{T_{1M}} = \frac{B\tau_C}{r^6}\left[\frac{7}{1 + (\omega_S\tau_C)^2} + \frac{3}{1 + (\omega_I\tau_C)^2}\right] \quad (2)$$

$$\tau_C \simeq \tau_S = \frac{\tau_{S0}}{(1 + \alpha\omega_S)^2} \quad (3)$$

where f is the fraction of water or methanol molecules bound to (or near) the metal ions at the catalytic sites, τ_M is the residence lifetime of these molecules at the sites, and $1/T_{1M}$ is the relaxation rate of the protons when the solvent molecules are bound. $B = 2.47 \times 10^{-32}$ cm^6 s^{-2} for Cu^{2+} ions (with $S = {}^1/_2$); r is some average separation of bound protons and paramagnetic metal ion; ω_S and ω_I are the Larmor frequencies (in angular units), respectively, of the Cu^{2+} and proton spins, and τ_C is a correlation time for the magnetic dipolar interaction that relaxes the protons. τ_C is taken as essentially equal to τ_S, the relaxation time of the paramagnetism of the Cu^{2+} ions, based on the values for τ_C that result from analysis of the data. τ_S is a function of magnetic field strength for many paramagnetic ions (McConnell, 1956; Bloembergen & Morgan, 1961), and the functional form of this dependence for Cu^{2+} ions in proteins is particularly complex (Koenig & Brown, 1973). In the present circumstances, τ_S appears to be independent of magnetic field below ~10 MHz[3] and only slightly field dependent at the highest fields we consider. This allows us to use the right-hand expression of eq 3, which is a simplification of a more general form and only incorporates the lowest order dependence of τ_S on field strength that may be anticipated. The parameter α is treated as adjustable. It is small in the sense that $\alpha\omega_S \simeq 1$ for magnetic fields of ~300 MHz in the present case. In the reduction of the data for water protons, α is first set equal to 0, and the values for τ_{S0}, r, and τ_M are obtained by a least squares comparison of data and theory. Then a value of α is chosen to refine the fit at

[2] Protein concentration is quoted throughout as molarity of monomeric units, though the protein is dimeric.

[3] Magnetic field strength is given in units of the Larmor precession frequency of protons in that field. The conversion is 4.26 MHz = 1 Oe = 1 G.

high fields, and the initial least-squares procedure is repeated. There is no a priori reason to justify the applicability of these equations in the present circumstances, particularly since the NMRD spectra of Cu^{2+}–proteins are so varied (Koenig & Brown, 1973); rather the data are described well by eq 1–3, and the procedure allows a quantitative comparison of methanol and water data.

In contrast to the Cu^{2+}-substituted enzyme, where the important aspects of the data are the paramagnetic contributions to the relaxation rates which can be related to a theory for paramagnetic relaxation, essentially all the results reported for the Co^{2+}-substituted enzyme are due to diamagnetic interactions of solvent protons with protein. We analyze these data accordingly, following Hallenga & Koenig (1976), by fitting the observed NMRD to the Cole–Cole (Cole & Cole, 1941) expression:

$$1/T_1 = (1/T_{1W}) + D + A\{\mathrm{Re}[1/(1 + i\nu/\nu_c)^{\beta/2}]\} = (1/T_{1W}) + D + \frac{A[1 + (\nu/\nu_c)^{\beta/2}\cos(\pi\beta/4)]}{1 + 2(\nu/\nu_c)^{\beta/2}\cos(\pi\beta/4) + (\nu/\nu_c)^{\beta}} \quad (4)$$

Here $1/T_{1W}$ is the relaxation rate of the solvent nuclei in the protein-free buffer, ν is the Larmor precession frequency of the protons in the applied magnetic field, and D, A, ν_c, and β are parameters to be determined from a fit of eq 4 to the data. "Re" stands for "the real part of".

For $\beta = 2$, eq 4 reduces to a constant plus a Lorentzian dispersive term

$$1/T_1 = 1/T_{1W} + D + A/[1 + (\nu/\nu_c)^2] \quad (5)$$

The dispersive part of the Cole–Cole expression, like the Lorentzian, drops to half its maximum value of A at $\nu = \nu_c$, the frequency at which the curve inflects. However, for $\beta < 2$, the Cole–Cole expression has a slower variation with ν than the Lorentzian. A simple two-site model of water exchange between bulk solvent and protein [shown to be inadequate by Koenig et al. (1975)] predicts a Lorentzian contribution to the relaxation (Koenig & Schillinger, 1969), whereas the experimental data are known to vary somewhat more slowly with ν (Fabry et al., 1970; Lindstrom & Koenig, 1974). The Cole–Cole expression should be regarded as a heuristic equation that has been shown to represent diamagnetic relaxation dispersion data very well; it has no a priori validity. We use it here to enable comparison of results for ADH under varying conditions, as well as with results for other diamagnetic protein solutions.

Results

Water Proton Relaxation for Cu^{2+} Substitution. Figure 1 shows solvent water proton NMRD data for a sample of Cu(c)Zn(n)-ADH, 1.53 mM monomer concentration, in 0.025 M Tes buffer and 0.2 M NaCl, pH 6.9; for the same sample after addition of, initially, 6 mM NAD^+ coenzyme and then an additional 6 mM NAD^+ to check that the changes had saturated; and for the sample with coenzyme after subsequent addition of 10 mM pyrazole, an inhibitor of the active zinc-containing enzyme. (The data have been corrected for dilution effects, which are at most 3%.) Subsequent optical spectra showed that the catalytic sites were 70% occupied. That the pyrazole bound was indicated by an immediate change in color from the deep blue of a type I copper center to a yellow-green characteristic of the Cu^{2+}–ADH–NAD^+–pyrazole complex (Maret et al., 1980). Also shown is the NMRD of an analogous solution of (diamagnetic) apo(c)Zn(n)-ADH, 1.65 mM monomer concentration. These last data have been linearly

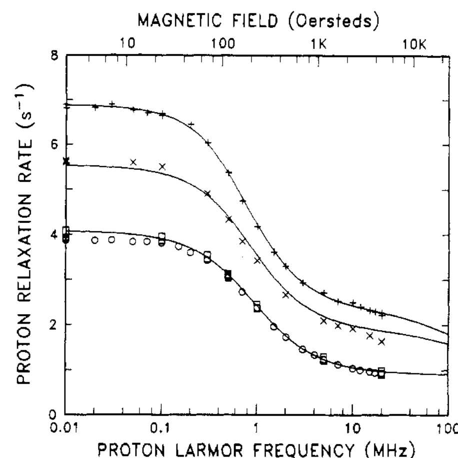

FIGURE 1: Dependence on magnetic field strength of the solvent water proton magnetic spin–lattice relaxation rate for (+) a sample of Cu(c)Zn(n)-ADH, 1.53 mM monomer concentration, in 0.025 M Tes buffer and 0.2 M NaCl, pH 6.9, and (×) the same sample after an initial addition of 6 mM NAD^+ (three points) and a subsequent addition of 6 mM NAD^+ (11 points) and (□) after addition of 10 mM pyrazole to form the ternary pyrazole–NAD^+–protein complex. Relaxation data for a sample of apo(c)Zn(n)-ADH, 1.65 mM monomer concentration, in the same buffer are also shown (○), after linear normalization to a concentration of 1.53 mM. All data were taken at 5 °C. The measured occupancy by Cu^{2+} ions of the catalytic sites was 70%. The lowest solid curve is a fit to the associated data by the usual methods for describing solvent relaxation in solutions of diamagnetic proteins (Hallenga & Koenig, 1976). The upper solid curve results from a least-squares comparison of the differences between its associated data and the lowest curve with the theory (eq 1–3) as discussed in the text. The middle solid curve is a fit to the differences between its associated data and the lowest curve obtained (mainly, but see text) by increasing the value of the resident lifetime of a water molecule obtained from the fit to the upper data, while keeping the values obtained for the other parameters fixed.

scaled from the measured values to adjust for the somewhat lower concentration of the Cu^{2+}–protein sample [cf. Hallenga & Koenig (1976)]; the validity of the linear correction was independently checked by comparison with NMRD data for samples of diamagnetic protein at other concentrations (see Table I).

The solid line through the upper data points results from a least-squares comparison of the paramagnetic contribution to the relaxation, taken as the difference between the uppermost and pyrazole-inhibited NMRD data, with eq 1–3. A smooth curve was first fit to the dispersion of the sample with added pyrazole, following Hallenga & Koenig (1976), and the appropriate subtractions were then made to obtain the paramagnetic contribution. The values found for the parameters are $\tau_{S0} = 5.5 \times 10^{-10}$ s, $r = 2.7$ Å, and $\tau_M = 4.5 \times 10^{-6}$ s. The value of α is such that $\alpha\omega_S = 1$ at a field of 300 MHz. To obtain f, we assumed that single water molecule was associated with every Cu^{2+} ion. The values derived for τ_S and α are independent of the value of f (they depend on the geometric properties of the NMRD spectra), whereas the values of r and τ_M are not.

The solid curve through the data points for the sample with only NAD^+ added was obtained by varying the value of τ_M derived above to obtain a best fit, without altering the values derived for the other parameters. The result, $\tau_M = 15 \times 10^{-6}$ s, shows that the data are entirely consistent with the view that only τ_M changes upon addition of coenzyme. More directly, considering the scatter in the data, it appears that the major effect of addition of NAD^+ is an increase in the residence lifetime of the water molecules on the Cu^{2+} ions. Subsequent addition of pyrazole presumably displaces the water [cf. Maret et al. (1980)], though the data, of course, could also be ex-

Table I: Results of Least-Squares Comparison of the Cole–Cole Expression (Equation 4) with Solvent Water Proton Relaxation Dispersion Data[a]

sample	figure	concn[a] (mM)	A_1 (s^{-1})	D_1 (s^{-1})	β	ν (MHz)	$2A/[C]^a$ (mM^{-1} s^{-1})	$2D/[C]^a$ (mM^{-1} s^{-1})
Co(c)Zn(n)-ADH	3	1.75	3.72	0.61	1.67	0.83	4.23	0.70
+NADH	3	1.74	4.13	0.49	1.71	0.77	4.74	0.56
+isobutyramide	3	1.72	4.01	0.35	1.70	0.76	4.66	0.41
Co(c)Zn(n)-ADH	4a, 5	2.77	5.72	0.92	1.66	0.85	4.14	0.66
at 25 °C	5	2.77	2.83	0.59	1.65	1.63	2.04	0.42
at 39 °C	5	2.77	2.12	0.44	1.49	2.5	1.53	0.31
apo(c)Zn(n)-ADH	4a	2.38	5.41	0.35	1.67	0.85	4.54	0.29
apo(c)Zn(n)-ADH	4b	1.65	3.42	0.19	1.69	1.00	4.14	0.23
Zn(c)Zn(n)-ADH	4b, 5	1.07	2.43	0.24	1.69	1.06	4.55	0.45
at 25 °C	5	1.07	1.19	0.07	1.68	2.0	2.22	0.13

[a] All results are for 4 °C unless indicated. Results are normalized for concentration. Though the concentration of ADH is quoted throughout as the concentration of monomers of 40 000-dalton nominal molecular weight, the normalization here is in dimeric units for ease of comparison with the results for other proteins given in the literature. [b] From Hallenga & Koenig (1976).

plained by a 20-fold or greater increase in τ_M.

By contrast to the results in Figure 1, in which it is seen that addition of pyrazole in the presence of coenzyme eliminates the paramagnetic contribution to the NMRD, addition of 25 mM pyrazole in the absence of coenzyme was found to reduce the paramagnetic effects only by ~20%. Increasing the pyrazole concentration to 75 mM produced no further change. Subsequent addition of coenzyme yielded a sample that reproduced the data of Figure 1 for the ADH–coenzyme–pyrazole ternary complex.

Methanol Methyl Proton Relaxation for Cu^{2+} Substitution. Experiments analogous to the above, but for solvent methanol methyl protons rather than for solvent water protons, were also performed. The problem that arises is that extensive dialysis of Cu(c)Zn(n)-ADH in solution against D_2O risks reduction of the Cu^{2+} ions to Cu^{1+}, with attendant loss of paramagnetism. The protein must therefore be maintained in the crystalline state during dialysis, where the possibility of reduction is greatly reduced (Maret et al., 1980). This in turn requires the presence of 20% *tert*-butyl alcohol (proton containing, in the present case), which is difficult to remove entirely when dissolving the crystalline protein. These considerations become a problem because the NMRD technique, unlike high-resolution nuclear magnetic resonance spectroscopy, does not distinguish among protons with different chemical shifts; all protons in the solvent are observed simultaneously and collectively.

Limited NMRD data are shown in Figure 2 for solvent protons for a 2.06 mM sample, nominally Cu(c)Zn(n)-ADH, prepared by first dialyzing the Cu(c)Zn(n)-ADH crystals against D_2O for over 120 h with four changes of buffer, and then dissolving the crystals in, and subsequently dialyzing the solution against, D_2O buffer for a few hours with continual stirring. On dissolving the crystals, we took care to remove as much of the *tert*-butyl alcohol as possible: the crystalline samples were centrifuged, the supernatant was removed, and the crystals were then washed twice with Tes–D_2O buffer and finally dissolved in the same buffer with 0.2 M KCl added. Buffer from the exterior of the dialysis bag showed no detectable trace of protons, whereas the signal from the sample indicated ~5% protons remaining in the mostly deuterated solvent. These protons are in all probability from solvent HDO molecules since their measured relaxation rates, after correction for the different concentration of Cu^{2+} ions and the altered diamagnetic contribution in deuteron-diluted solvent (Koenig et al., 1978b), agree with the results in Figure 1. There is the remote possibility that the signals are from *tert*-butyl alcohol remaining from the crystalline state; however, we consider this unlikely because of the care taken to eliminate this possibility (see above) and the lower paramagnetic contribution expected since the ion–proton distance of metal-bound *tert*-butyl alcohol would be comparatively large. In either case, this is not a substantive point in our conclusions as presented under Discussion.

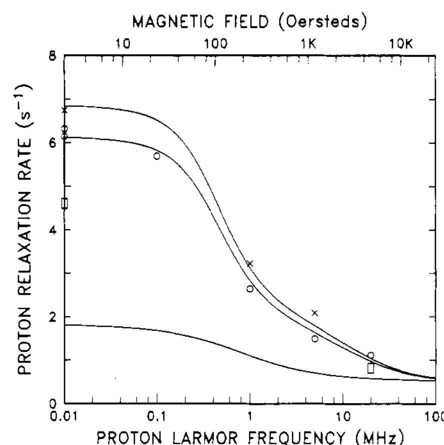

FIGURE 2: Dependence on magnetic field strength of the solvent proton magnetic spin–lattice relaxation rate for Cu(c)Zn(n)-ADH, nominally 2.06 mM, (×) in Tes–D_2O buffer, 0.2 M KCl, and 2.5 M CH_3OD, (O) after addition of 10 mM NAD^+, and (□) after addition of 8 mM pyrazole (but see text). The lowest solid curve results from a fit to methanol methyl proton relaxation data for a comparable sample of ADH with no paramagnetic contribution, 1.88 mM monomer concentration, normalized to the concentration of the paramagnetic sample. All data were taken at 5 °C. The middle solid curve results from a least-squares comparison of the differences between its associated data and the lowest curve with the theory (eq 1–3) with only f, τ_M, and α taken as adjustable parameters (see text). The upper solid curve was obtained from the preceding results by varying only f (i.e., essentially adjusting only the relative affinities of the Cu^{2+} ions for waters and methanol as ligands).

CH_3OD was subsequently added to the above sample to produce solvent 2.5 M (~10%) in methanol. The signal intensity increased by a factor 2.5, consistent with the estimate of 5% protons remaining after dialysis. Limited NMRD data for this case are also shown in Figure 2. The relaxation rates, averages over the two classes of solvent protons, are essentially unchanged upon addition of methanol even though the resonance signal is contributed roughly equally by (presumably) water and methanol protons. Analysis of the details of the magnetization decay showed it to be exponential, indicating that the signal does indeed correspond to all the solvent protons relaxing at a common rate, or at rates that are very similar in value.

Addition of NAD^+ lowers the relaxation rates somewhat, as seen in Figure 2, much as for water protons (Figure 1). Addition of 8 mM pyrazole to this sample produced no color

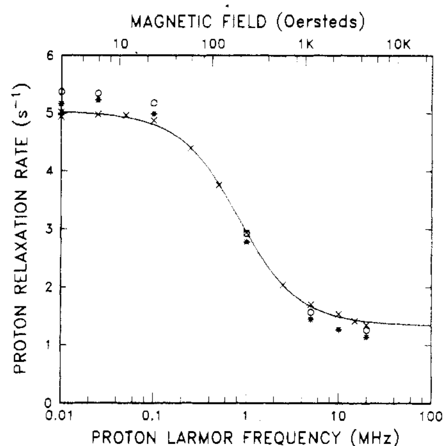

FIGURE 3: Spin-lattice relaxation rate of solvent water protons as a function of magnetic field strength for a 1.75 mM sample of Co(c)Zn(n)-ADH at 4 °C (×) in 0.025 M Tes buffer and 0.5 M NaCl, pH 7.04, (O) after addition of 1.6 mM NADH, and (*) after subsequent addition of 1.6 mM isobutyramide. The solid curve derives from a least-squares comparison of the data (×) with eq 4, which describes relaxation by diamagnetic proteins.

change, contrary to expectations, and caused extensive precipitation of protein. The observed reduction in relaxation rates, though anticipated by analogy with the results in Figure 1, could well be due to loss of protein by precipitation. Relaxation data for methyl protons for the native enzyme are also shown in Figure 2.

In anticipation of arguments to be made under Discussion, we consider that methanol and water compete for the same ligands of the Cu^{2+} ions with approximately equal affinities in making the initial comparisons of the data with theory. This would mean that in a 2.5 M methanol solution, 5% of the Cu^{2+} ions are liganded to methanol and the remainder to water (D_2O). Using the value $r = 3.3$ Å as a reasonable average for the three methyl protons and τ_{S0} obtained from the fit of the data in Figure 1, we obtained the solid line through the NAD^+-protein data of Figure 2 by adjusting f, τ_M, and α for a best fit to the paramagnetic contribution to the relaxation, in this case taken as the difference between the data for the NAD^+-protein sample and that for the native enzyme. The value obtained for f corresponds to a dissociation constant for methanol about twice that of water, and the value for τ_M, 1×10^{-6} s, is too small to contribute significantly in eq 1. This suggests that the effect of NAD^+ may be mainly on the relative affinities of the Cu^{2+} ion for water and methanol and, accordingly, the solid curve through the upper set of data points was obtained by increasing f rather than altering τ_M.

Water Proton Relaxation for Co^{2+} Substitution. Figure 3 shows solvent water proton NMRD data for a 1.75 mM sample of Co(c)Zn(n)-ADH, in 0.025 M Tes buffer and 0.5 M NaCl, pH 7.04 and limited NMRD data for the same sample after addition of 1.6 mM NADH coenzyme and after subsequent addition of 1.6 mM isobutyramide inhibitor. The data are corrected for the effects of the 1.7% dilution caused by additions of first coenzyme and then inhibitor.

Additions of coenzyme and then inhibitor produced the expected changes in color of the sample, from light blue to green and then to yellow green (Dietrich et al., 1979; Andersson et al., 1980), indicating complex formation. The solid curve of Figure 3 results from a least-squares comparison of the data with eq 4; the values derived for the four parameters are given in Table I. Note that addition of coenzyme makes the enzyme appear slightly "heavier" (Hallenga & Koenig, 1976); the high-field rates decrease somewhat, the low-field rates increase, and ν_c decreases (cf. Table I). Addition of

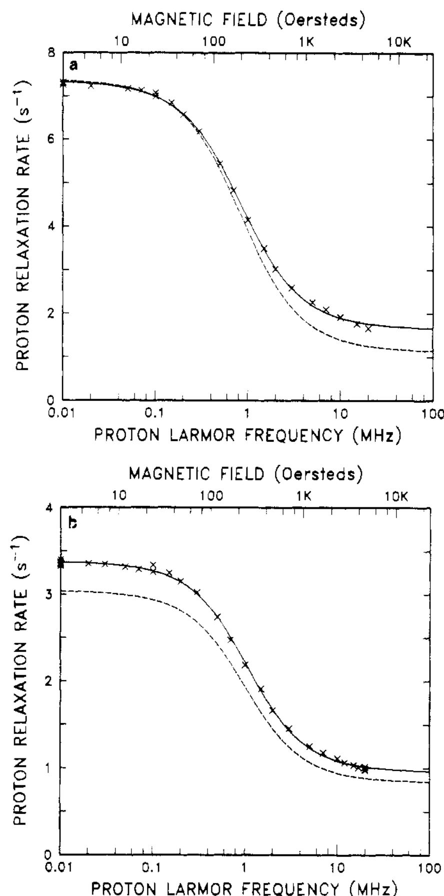

FIGURE 4: (a) Spin-lattice relaxation rate of solvent water protons as a function of magnetic field strength for (×) a 2.77 mM sample of Co(c)Zn(n)-ADH, compared with (dashed curve) a 2.38 mM sample of apo(c)Zn(n)-ADH, the latter data scaled linearly to compensate for the difference in protein concentrations of the two samples. The solid curve derives from a least-squares comparison of the data (×) with eq 4, which describes relaxation by diamagnetic proteins. (b) Similar to (a) except that the upper data are for 1.07 mM Zn(c)Zn(n)-ADH and the lower for 1.65 mM apo(c)Zn(n)-ADH, again scaled for the difference in protein concentrations. In each case, the data are at 4 °C for samples in 0.025 M Tes buffer and 0.5 M NaCl, pH 7.04.

inhibitor, on the other hand, decreases the relaxation rates slightly at all fields, by reducing both A and D (eq 4) slightly. Consideration of only the high-field data might suggest that the changes are caused by successively decreasing paramagnetic contributions; however, such a view cannot be readily reconciled with the entire NMRD spectra.

Figure 4a,b show comparisons of NMRD spectra for (native) Zn(c)Zn(n)-ADH and Co(c)Zn(n)-ADH and two samples of apo(c)Zn(n)-ADH. The protein concentrations of the four samples differ so that the comparisons are not quite straightforward. One demetalized sample had a concentration close to, but lower than, that of the Co^{2+} sample; the data for it were normalized to the concentration of the Co^{2+} sample by linear extrapolation before plotting. Conversely, the second demetalized sample had a higher concentration than the Zn^{2+} sample; its spectrum was normalized in this case to the concentration of the Zn^{2+} sample. The values for the four parameters of eq 4 for each sample are listed in Table I. From these results and from what is known about the concentration dependence of diamagnetic relaxation rates, it is seen that the linear extrapolation underestimates differences between the curves in Figure 4a,b. Thus, the native enzyme has a greater relaxivity (relaxation rate per mole of protein) than the partially demetalized protein, and the (paramagnetic) Co^{2+}-substituted enzyme has a lesser relaxivity than the native

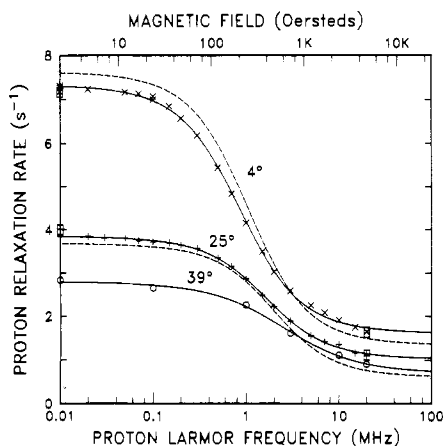

FIGURE 5: Spin–lattice relaxation rate of solvent water protons as a function of magnetic field strength for a 2.77 mM sample of Co(c)Zn(n)-ADH: first (×) at 4 °C and then at (+) 25 and (○) 39 °C. The dashed curves represent similar data for 1.07 mM Zn(c)-Zn(n)-ADH at 4 and 25 °C, scaled to compensate for the difference in protein concentrations of the two samples. The data indicated by (□) were obtained after reaching 39 °C, as a check. The solid curves derive from a least-squares comparison of the data for Co(c)Zn(n)-ADH with eq 4, which describes relaxation by diamagnetic proteins. The data are for samples in 0.025 M Tes buffer and 0.5 M NaCl, pH 7.04.

enzyme over most of the range of magnetic field. However, there are slight differences in the relatively large diamagnetic effects at low fields that are manifested as large fractional variations in the relaxation rates at high fields.

To allow for the possibility that water bound to the Co^{2+} ions exchanges with solvent too slowly to contribute observably to relaxation, we obtained NMRD spectra of the Co^{2+}-substituted enzyme at two higher temperatures. Figure 5 shows these data, together with a comparison of the results with eq 4 which, as is seen, provide an adequate description of the data at all temperatures. The values for the parameters are listed in Table I. (Note that a few points were taken at 4 °C after those at the highest temperature were measured, to ascertain that the properties of the protein were not irreversibly altered by the high temperature.) Also shown for comparison are results for a sample of native enzyme, that used for the data of Figure 4b, obtained by normalizing the raw data to the higher concentration of the Co^{2+}-substituted enzyme. No qualitative changes appear in the NMRD data to suggest the onset of a paramagnetic contribution at the higher temperatures.

Ancillary Experiments. To allow for the (remote) possibility that monovalent Cl^- ions might bind to the Co^{2+} ions and displace water, given the relatively high NaCl concentration used to stabilize the protein in solution [cf. Dahl & McKinley-McKee (1980)], we repeated NMRD measurements on a sample with Na_2SO_4 in the buffer, at the same ionic strength as the NaCl initially used. The major anion is now divalent, and there is ample reason to believe that even if monovalent anions bind, divalent anions would not (Koenig et al., 1980). The measure relaxivities were unaltered in these experiments.

A series of NMRD measurements were made for solvent methanol methyl protons in samples dialyzed extensively against D_2O, after which CH_3OD was added to make the solvent ~2 M methanol. The thought was that methanol would certainly bind to the Co^{2+} ions, even if for some reason water did not, so that the paramagnetism of the Co^{2+} ions might be detected through its contribution to the relaxation of the methyl protons. These experiments were preliminary to the analogous experiments on the Cu^{2+}-substituted protein reported above and were perforce more rudimentary. Coenzyme and inhibitor were also added, but from stock solutions in nondeuterated solvent, so that the ratio of the concentrations of water protons to methyl protons was not constant in these experiments. Although a quantiative analysis of these data is difficult, and not warranted under the circumstances, the qualitative results was that there was no indication of any contribution to the methyl proton relaxation that could be attributed to paramagnetism of the Co^{2+} centers.

Finally, measurements were made on a sample of apo(c)-Zn(n)-ADH to which Co^{2+} ions were added in solution. The results, more germane to the following paper (Andersson et al., 1981), were that it requires several hours for the added ions to occupy the catalytic sites, as judged by the relaxation rates (which were those of the aquo ions plus that of the apo(c)Zn(n)-ADH) and by the slow change of color of the solution.

Discussion

Water Proton Relaxation of Cu^{2+}-Substituted Enzyme in Undeuterated Solvent. The contribution of the Cu^{2+} ions to the solvent proton relaxation (Figure 1) is in some sense classic. The data can be described very well by the simple (indeed, oversimplified) theory of relaxation, with α quite small and therefore τ_S essentially independent of field; the value of r is very close to the value of 2.8 Å expected for a water ligand of Cu^{2+} ions; the paramagnetic component of the NMRD spectra is eliminated by addition of pyrazole in the presence of coenzyme under conditions for which the active, native enzyme would be inhibited and under which changes in the visible spectra indicate that the pyrazole is interacting with the Cu^{2+}-substituted protein (Maret et al., 1980). However, the value derived for τ_{S0} is unusually short compared to that for a nonblue Cu^{2+}–protein complex; typical values are almost 2 orders of magnitude longer (Koenig & Brown, 1973). Consistent with this short τ_{S0} is the fact that no electron spin resonance lines are observed at room temperature for Cu(c)Zn(n)-ADH solutions (Maret et al., 1980, and unpublished results).

This is the first study, by NMRD, of a deep blue Cu^{2+}–protein for which, apparently, a solvent molecule is a ligand of the metal ion and where thiolate forms two of the remaining ligands of the (presumed) tetrahedrally coordinated Cu^{2+} ion. Traditional wisdom has it that such deep blue, so-called type I, centers result from ligand fields with distorted geometry that delocalize the Cu^{2+} orbitals onto the thiolate centers; this, in turn, suggests coordination exclusively by residues of the protein, in contrast to the case here [cf. Coleman et al. (1978)]. Nonetheless, the unusually short correlation time (for Cu^{2+}) indicates an unusually strong coupling of the electronic spin of the Cu^{2+} ion to the protein structure, in accord with the traditional ideas regarding the nature of the ligand fields of blue Cu^{2+} centers. There is no inconsistency here, however, since the coupling of the electronic spin to the lattice is through the spin–orbit interaction associated with the orbital of the unpaired spin. Since the spin–orbit interaction is proportional to the square of the nuclear charge and to the inverse of the radial quantum number, sulfur should produce a 3-fold greater spin–orbit interaction than oxygen or nitrogen ligands. This effect, in turn, enters quadratically into the theory for τ_{S0}. The increased spin–orbit interaction due to sulfur, combined with the increased delocalization of the orbital (charge transfer) onto the sulfur as indicated by the blue color, should account for the large electronic relaxation rate of the Cu^{2+} ions observed here.

Though the data in Figure 1, for which NAD^+ and then pyrazole were added, appear straightforward, the converse

experiment (in which pyrazole was added first) requires some comment. Pyrazole added in saturating amounts, at concentrations greater than those for which Maret et al. (1980) identified a well-defined binary pyrazole–protein complex by both electron spin resonance and optical measurements, decreases the paramagnetic part of the water relaxation by only ~20%. One inference is that, in the absence of coenzyme and the presence of pyrazole, the Cu^{2+} ions of Cu(c)Zn(n)-ADH may be pentacoordinated, including both a water molecule and a pyrazole from solution as ligands. This configuration is consistent with the fact that Maret et al. (1980) have termed this binary complex "a novel type of Cu^{2+}–protein" and that they observe superhyperfine structure on the Cu^{2+} electron spin resonance signal due to the nitrogen of the pyrazole. Addition of coenzyme to this complex must, of course, induce the known conformation change associated with binding of coenzyme and presumably restricts the geometry in the region of the catalytic site so that Cu^{2+} ion is once again tetracoordinated.

Considerations of pentacoordination also relate to the data of Figure 1 in the absence of pyrazole. Though we assumed that there was a single water ligand of Cu^{2+} ions in the absence of coenzyme, with a lifetime that increased upon addition of coenzyme, given the uncertainties of relaxation theory (Koenig & Brown, 1973; Koenig, 1978), the possibility that binding of coenzyme reduces the number of water ligands of the Cu^{2+} ions from two to one is not precluded by the present data. A change in number of water ligands by a factor of 2, without altering other parameters, only alters r by a factor of about $2^{1/6}$, corresponding to ~12%. However, there is no need to introduce such effects on the basis of the NMRD data alone. Indeed, if addition of coenzyme were to alter the coordination number of the Cu^{2+} ions, then to explain the NMRD results would require consideration of the possibility of different values of τ_M for each water and changes in τ_{S0} and α upon coenzyme binding as well. It appears more reasonable at present to take the view that, since coenzyme blocks one of two channels from solvent to the catalytic site, leaving a hydrophobically lined tube as the remaining access (Brändén, 1977), the drop in relaxation rate upon addition of coenzyme (Figure 1) is indeed caused by an increase in the mean lifetime of a single water ligand on each of the Cu^{2+} ions.

Though the above considerations may clarify the issue of access of solvent water to Cu(c)Zn(n)-ADH and its binary and ternary complexes with coenzyme and inhibitor, there is no suggestion in these data that indicates why the protein is inactive enzymatically. This requires consideration of the interaction of the protein with methanol.

Methanol Methyl Proton Relaxation of the Cu^{2+}-Substituted Enzyme in Deuterated Solvent. The data for this case (Figure 2) are somewhat limited; nonetheless, the major conclusion that can be drawn from these data is unequivocal: methanol has an affinity for Cu(c)Zn(n)-ADH comparable to that of water, so that for solvent that is half water and half methanol, roughly half the Cu^{2+} ions would be liganded to water and the remainder to methanol. This corresponds to a dissociation constant for methanol of the order of 30 M, compared with about 50–100 mM for the native enzyme in the presence of coenzyme (Brooks & Shore, 1971). Thus from the point of view of enzymology, the Cu^{2+}-substituted enzyme appears inactive because substrate binds too weakly, even though coenzyme binds. In essence, the activity is lowered 100-fold or more because of the weak binding of substrate.

It should be noted that the concentrations of methanol used here (2.5 M or ~10% by volume) do not denature the protein. Excess alcohol causes the protein to crystallize; the crystallized enzyme is active, and active solutions of enzyme can be obtained by removing the excess alcohol after months of storage (Zeppezauer et al., 1967; Söderberg et al., 1970).

The foregoing conclusions follow from consideration of the various possible situations that can explain the data in Figure 2. Recall that about half the resonance signal is contributed by methanol methyl protons and half by other protons, most likely water protons from HDO, whereas only 5% of the solvent molecules are methanol. If the affinity of methanol for Cu(c)Zn(n)-ADH was comparable to that for the native enzyme, then the majority of the water molecules, deuterated or not, would be displaced from the Cu^{2+} ions in the presence of 2.5 M methanol and the water proton relaxation rates would become very small. Since the observed relaxation rates remain high, the methanol would have to be in rapid exchange. However, this situation implies two decay rates for the observed magnetization, one rapid and one slow. Operationally, the slower one would either appear as an increased base line, to about half the maximum signal, or else not contribute to the magnetization at all, causing a reduction in the signal amplitude. Neither of these conditions agrees with the observations. Conversely, if methanol bound much more weakly than water, the foregoing argument would obtain, but with the roles of water and methanol reversed. The data are only consistent with water and methanol interacting essentially equivalently with the Cu^{2+} ions at the catalytic sites.

These conclusions are not influenced by the relative paucity of data in Figure 2, by possible uncertainties in the diamagnetic contribution to the NMRD spectra, or by the uncertainties in the theory. If 2.5 M methanol saturated all the Cu^{2+} ions, the observed relaxation rates would be 20-fold greater; recall that the reasonable values obtained for r depend on a value for f (eq 1) that assumes equivalent binding for water and methanol. Thus the large quantitative difference between the observed and expected values of relaxation rates for the two conditions, weak and strong binding of methanol, leads to the unequivocal qualitative conclusion that enzymatic activity is not observed for Cu(c)Zn(n)-ADH because the coenzyme–protein complex essentially does not bind substrate.

There is every reason to have anticipated this conclusion; water and methanol are much alike in many ways. Certainly, the nature of the Cu–O bond to the oxygen of either water or methanol should depend little on whether the oxygen is bonded to a proton or a methyl group. This is consistent with the work of Luz & Meiboom (1964a,b), who studied the preferential solvation of Ni^{2+} and Co^{2+} aquoions in methanol–water solutions at fairly low temperatures. Their results are consistent with comparable solvation of the Ni^{2+} and Co^{2+} ions by either type of solvent molecule at room temperature and, by extension, for Cu^{2+} as well. Moreover, analogous results have recently been found for competition between methanol and water for the metal ligand of Co^{2+}-substituted carbonic anhydrase (Jacob et al., 1980). Thus it appears that coenzyme has an important role in native ADH other than to serve as a reducing agent during the catalytic oxidation of alcohols. It must also provide the required selectivity of the catalytic site for alcohol substrates compared to water, a role it apparently cannot perform for all species of metal ion at the catalytic site.

Paramagnetic Effects in the Co^{2+}-Substituted Enzyme. It is instructive first to tryp to estimate the anticipated magnitude of the paramagnetic contribution of bound Co^{2+} ions to solvent water proton relaxivity. The closest model is probably Co^{2+}-substituted bovine carbonic anhydrase, for which extensive data exist (Koenig et al., 1980; Wells et al., 1979). The

similarity of the environments of the metal ions in these two enzymes has been noted by Argos et al. (1978). The ligand configuration of Co^{2+} ions in both (active) enzymes is tetrahedral; however, the metal ion has three histidyl nitrogen ligands in carbonic anhydrase, whereas it has one histidyl and two cysteinyl ligands in ADH. For Co^{2+}-substituted carbonic anhydrase, the paramagnetic contribution to the relaxivity is 1 mM^{-1} s^{-1} at 0.02 MHz and 0.7 mM^{-1} s^{-1} at 20 MHz [Wells et al. (1979), Figure 3]. If the contribution were the same for Co^{2+}-substituted ADH, the paramagnetic contribution to the data in Figure 3 would be substantial; it would add $\sim$1.8 s^{-1} at low fields and 1.0 s^{-1} at high fields. The changes upon addition of inhibitor would be of this order as well. However, in none of the experiments reported here is there any evidence of a contribution greater than, say, $\sim$0.2 s^{-1} that could be attributed to paramagnetism. This is a conservative estimate of the maximum paramagnetic contribution that could remain undetected, given the small variations in the diamagnetic relaxivity with sample composition.

In our view, the reason for the lack of an observable paramagnetic contribution to the relaxivity is that, although solvent has access to the Co^{2+} ions, the magnetic interaction is too small to produce an observable effect. This implies that the correlation time τ_S for the interaction between the solvent protons and the paramagnetic ions is shorter than in Co^{2+}-substituted carbonic anhydrase by at least 1 order of magnitude, corresponding to a greatly increased interaction of the spin of the Co^{2+} ions with the ligands of the surrounding protein in ADH. This is not surprising in that the mechanisms that determine the Co^{2+}–protein interaction all involve the spin–orbit interaction of the paramagnetic orbitals of the Co^{2+} ions. These, of course, overlap the sulfur ligands and, because of the greater spin–orbit interaction in heavier elements, increase the magnetic relaxation rate of the paramagnetism of the Co^{2+} ions. Completely analogous conclusions were reached above for the Cu^{2+} centers, for which paramagnetic contributions to solvent relaxation were observed. In that case, the effect of the sulfur ligands was to decrease τ_S by $\sim$2 orders of magnitude. An alternate explanation that solvent molecules (either water or methanol) have no access to the Co^{2+} ions is unsupportable, judging from analogies with relaxation results for the Cu^{2+}-substituted ADH, X-ray data for the native enzyme (Brändén et al., 1975), and the fact that Co^{2+}-substituted ADH is enzymatically active (Sytkowski & Vallee, 1978; Maret et al., 1979).

These conclusions are at variance with those of Sloan et al. (1975), who reported the relaxation behavior of ethanol methyl protons (which would be much farther from the Co^{2+} ions than methanol methyl protons) for solutions of both the fully Co^{2+}-substituted enzyme and what they thought was ADH with Co^{2+} ions specifically substituted for Zn^{2+} at the catalytic site. They attributed the small changes that they observed at 100 MHz to paramagnetic effects, changes that are well within the variation of the diamagnetic contributions upon metal ion substitution, as we have seen. Burton et al. (1979) have also commented on the small changes observed. Moreover, it is now known that in the work of Sloan et al. (1975) the specifically substituted Co^{2+} ions were at the structural and not the catalytic sites (Andersson et al., 1980; Maret et al., 1979). The data of Sloan et al. (1975), when reexamined in the light of this new information, are completely consistent with the results reported here. In particular, they noted almost no change in relaxation between their hybrid enzyme (with Co^{2+} ions at the noncatalytic sites) and their fully substituted enzyme. However, the extensive model building that Sloan et al. (1975) do, including the computation of well-defined distances and a commitment to second-sphere coordination for substrate at the Co^{2+} ions, must be reconsidered since the conclusions refer to Co^{2+} ions at the noncatalytic sites. Finally, our conclusions also resolve the apparent conflict between the results of Sloan et al. (1975) and the X-ray data [cf. Brändén & Eklung (1978), particularly the recorded discussion following that paper].

Summary. We find that solvent water and methanol molecules bind comparably to the Cu^{2+} ions of ADH with Cu^{2+} ions substituted specifically for Zn^{2+} ions at the catalytic sites. Addition of coenzyme does not increase the relative affinity of the site for methanol, as it does for the native enzyme and as is necessary for effective catalytic activity. Thus Cu(c)Zn(n)-ADH shows little, if any, activity (Maret et al., 1980) because it does not bind substrate effectively. Whether it has any intrinsic catalytic activity, i.e., k_{cat} comparable to that of the native enzyme, remains to be established.

Addition of coenzyme lessens, and subsequent additional pyrazole eliminates, the contribution of the Cu^{2+} ions to the water proton relaxivity rate. By contrast, pyrazole alone only slightly reduces the relaxation rates, suggesting pentacoordination of the Cu^{2+} ions in the absence of coenzyme.

The relaxation behavior of the paramagnetic blue Cu^{2+} centers in this protein is unlike that of other blue proteins that have been studied. Both methanol and water protons are relaxed to an extent that is consistent with inner-sphere or Cu^{2+}–ligand coordination. This contrasts with results for azurin and ceruloplasmin (Koenig & Brown, 1973) for which water proton relaxation studies have shown the blue Cu^{2+} centers to be well below the surface of the protein and inaccessible to solvent. Moreover, the present data yield an unusually short (for Cu^{2+} ions) correlation time for the paramagnetic interaction ($\sim 10^{-11}$ s), indicating a stronger than usual coupling of the spin of the Cu^{2+} ions to the protein. We associate this with delocalization of the paramagnetic orbitals onto the two cysteine sulfur ligands, as inferred from the blue color, and the concomitant large spin–orbit interaction due to the greater nuclear charge of sulfur compared to (the usual) oxygen or nitrogen ligands.

An assiduous search for a paramagnetic contribution to the magnetic relaxation rate of solvent protons in solutions of horse-liver alcohol dehydrogenase with Co^{2+} ions substituted specifically for Zn^{2+} ions at the catalytic site has yielded no measurable contribution. There are small differences in the diamagnetic ontribution to the relaxation rates among the native, partially demetalized, and Co^{2+}-substituted proteins, with and without added coenzyme and inhibitor, that may result from small conformational differences. That these effects are indeed variations of the diamagnetic contributions and not attributable to paramagnetism of the Co^{2+} ions, however, could only be demonstrated by analyzing the complete NMRD spectra; i.e., by measuring relaxation rates over a wide range of magnetic field values, including field strengths well below those at which magnetic resonance experiments are usually done. The relaxation rates for solutions of the paramagnetic enzyme are lower than those for the native enzyme over most of the range of magnetic field; ironically, however, small positive effects persist at high fields, after the major diamagnetic contributions have dispersed away, that appear as fairly large fractional effects, even though they are small in absolute value. As a result, data taken only at high fields can be readily misinterpreted (Boccalon et al., 1978; Sloan et al., 1975) as paramagnetic contributions from interactions of solvent protons with the Co^{2+} ions in solutions of the

Co^{2+}-substituted enzyme that can in turn be eliminated by inhibitors.

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