a model 1280 computer and operated at frequencies of 361.062 MHz for proton and 90.793 MHz for ^{13}C observation. The pulse sequence employed for the experiment was essentially that of Freeman[27] modified to deliver a composite 180° pulse.[40] Pulse widths were 30 μs for carbon and 32 μs for proton via the decoupler coils. Fixed delays around the acquisition pulse (Δ_1 and Δ_2) were set to 3.0 and 2.5 ms (respectively) with a spectral width of ±833 Hz for proton and ±3425 Hz for carbon, with phase cycling to provide the equivalent of quadrature data in both dimensions. The initial $S(t_1,t_2)$ data matrix was generated using 256 × 2K blocks of data followed by processing in the usual fashion. The experiment was performed on a sample prepared by dissolving 200 mg of **1** in approximately 2 mL of deuteriochloroform. Accumulation of the initial $S(t_1,t_2)$ data matrix required approximately 3 h. The contour plot (Figure 1) was prepared by using four contour levels. The proton reference spectrum was the projected sum of the data matrix through the F_1 dimension[36] and the carbon reference spectrum was the projection through the F_2 dimension.

(40) Levitt, M. H.; Freeman, R. *J. Magn. Reson.* **1981**, *43*, 65-80.

Acknowledgment. This paper is dedicated to Professor William von Eggers Doering on the occasion of his 65th birthday. His excitement about the science of chemistry has been continuously transmitted to M.R.W. for the past 25 years. In some small way, this enthusiasm has also been transmitted to all of the other authors of this paper. Several of the authors, G.E.M., M.A., A.J.W., and M.R.W. wish to thank the Robert A. Welch Foundation for its generous support through Grants No. E-792, E-745, E-744, and E-183. The authors also acknowledge the support of the National Science Foundation in the form of Grants No. CHE-7506162 and CHE-7818723, which provided the funds for the acquisition of the XL-100 spectrometer system at the University of Houston and for the establishment and operation of the South Carolina Regional NMR Facility, respectively. Lastly, G.E.M. also acknowledges the partial support of this work by the University of Houston Limited Grant in Aid Program.

Registry No. 1, 22551-45-7.

^1H NMR Investigation of the Active Site of Cobalt(II)-Substituted Liver Alcohol Dehydrogenase

I. Bertini,*† M. Gerber,‡ G. Lanini,† C. Luchinat,† W. Maret,‡ S. Rawer,‡ and M. Zeppezauer‡

Contribution from the Department of Chemistry, University of Florence, Florence, Italy, and Fachbereich 15, Analytical and Biological Chemistry, University of Saarland, Saarbrücken, F.R.G. *Received June 6, 1983*

Abstract: The pH dependence of the ^1H NMR spectra of active-site specifically substituted cobalt(II) horse liver alcohol dehydrogenase and its complexes with NAD^+ and NADH are reported. The ^1H NMR signals of the cysteine and the histidine ligands are well shifted from the diamagnetic position. The δ-NH of histidine 67 probably deprotonates with a pK_a of 9.0 ± 0.2; in the complex with NAD^+ the same group exhibits a pH-dependent shift without deprotonation with a pK_a of 8.3 ± 0.2. The complex with NADH is pH independent up to pH 9.3. Both the ^1H NMR and near-IR spectra indicate that no major change in the coordination sphere of the catalytic metal ion occurs upon binding coenzyme. The results suggest the participation in catalysis of groups not considered in previously proposed mechanisms.

Liver alcohol dehydrogenase (LADH, EC 1.1.1.1) is a dimeric zinc enzyme of molecular weight 80 000, which catalyzes the reversible oxidation of alcohols. Each subunit contains two zinc ions. One zinc is essential for the substrate binding and activation while the other presumably plays a structural role. X-ray crystallographic studies of the native enzyme[1] and the cobalt(II)- and cadmium(II)-substituted enzymes[2,3] have shown that the catalytic metal ion is bound to histidine 67, cysteine 46, and cysteine 174 in a distorted tetrahedral geometry, the fourth ligand being a solvent water molecule. At least four protonation steps with pK_a values of 6.4, 7.6, 9.2, and 11.2 have been shown to be important for the catalytic action of the enzyme by kinetic studies.[4,5] The assignments of these ionizations to functional groups of the enzyme given in the literature do not have a firm physical basis. The pK_a of 9.2 has been attributed to the deprotonation of the zinc-coordinated water in the free enzyme and the pK_a's of 7.6 and 11.2 to the deprotonation of the same moiety in the binary complexes with oxidized and reduced coenzymes, respectively.[5,6] The interaction of the positive charge of the nicotinamide ring of NAD^+ with the catalytic zinc ion was proposed to cause the decrease of the pK_a of metal-bound water.[7] It should be noted, however, that the pH dependence of the coenzyme dissociation processes for the enzyme depleted of the catalytic zinc ion is similar to that of the native enzyme.[8] The pK_a of 6.4 has been tentatively assigned to the deprotonation of the zinc-coordinated alcohol on the basis of an apparent Brønsted relationship between the pK_a's of the free and bound alcohols.[9]

Cobalt(II) can be selectively substituted for zinc(II) in the catalytic sites of LADH.[10] The resulting derivative (Co(c)$_2$Zn(n)$_2$-LADH) retains catalytic activity although differences in the rate constants of elementary steps along the catalytic pathway have ben observed.[11,12] The electronic spectra of Co-

†University of Florence.
‡University of Saarland.

(1) Brändén, C. I.; Jörnvall, H.; Eklund, H.; Furugren, B. In "The Enzymes", 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. 11, pp 103-140.
(2) Schneider, G.; Eklund, H.; Cedergren-Zeppezauer, E.; Zeppezauer, M. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5289.
(3) Schneider, G. Dissertation, Universität des Saarlandes, Saarbrücken, 1983.
(4) Klinman, J. P. *Crit. Rev. Biochem.* **1981**, *10*, 39.
(5) Kvassman, J.; Pettersson, G. *Eur. J. Biochem.* **1979**, *100*, 115.
(6) Andersson, P.; Kvassman, J.; Lindström, A.; Oldén, B.; Pettersson, G. *Eur. J. Biochem.* **1981**, *113*, 425.
(7) Theorell, H. "The Harvey Lectures"; Academic Press: New York, 1967; series 61, pp 17-41.
(8) Dietrich, H.; MacGibbon, A. K. H.; Dunn, M. F.; Zeppezauer, M. *Biochemistry* **1983**, *22*, 3432.
(9) Kvassman, J.; Petterson, G. *Eur. J. Biochem.* **1980**, *103*, 565.
(10) Maret, W.; Andersson, I.; Dietrich, H.; Schneider-Bernlöhr, H.; Einarsson, R.; Zeppezauer, M. *Eur. J. Biochem.* **1979**, *98*, 501.
(11) Dunn, M. F.; Dietrich, H.; MacGibbon, A. K. H.; Koerber, S. C.; Zeppezauer, M. *Biochemistry* **1982**, *21*, 354.

0002-7863/84/1506-1826$01.50/0

$(c)_2Zn(n)_2$–LADH and of its binary complex with NAD^+ are pH dependent with pK_a values around 8 and 9, respectively.[13] These values have been related to the pK_a's of 7.6 and 9.2 reported for the Zn_4–enzyme by kinetic studies.[4,5] In contrast, the electronic spectra of the binary complex of $Co(c)_2Zn(n)_2$–LADH with NADH are pH independent in the pH range from 6 to 9.[13]

Recently, proton NMR spectroscopy has been shown to be a powerful tool to investigate the coordination sphere of the catalytic cobalt(II) ion in cobalt-substituted zinc enzymes.[14-16] The technique uses the relatively small relaxing properties of the high-spin cobalt(II) and its ability to induce relatively large isotropic shifts. We have therefore undertaken an NMR investigation of $Co(c)_2Zn(n)_2$–LADH and of its binary complexes with NAD^+ and NADH with the aim of characterizing the origin of the observed pH-dependent transitions. The electronic absorption and CD spectra have also been recorded down to the near-infrared region to interpret the observed d–d transitions in terms of coordination geometry.

Materials and Methods

Horse liver alcohol dehydrogenase (LADH, EC 1.1.1.1), NAD^+, and NADH (grade I) were purchased from Boehringer/Mannheim F.R.G. All other reagents were of analytical or spectroscopic grade. The active-site specifically substituted cobalt(II) horse liver alcohol dehydrogenase was prepared, manipulated, and characterized as described in ref 10. $Co(c)_2Zn(n)_2$–LADH was dissolved in 33 mM Mes (pH 6–7), Tes (pH 7–8.5), or Taps (pH 8.5–9.5) buffer in the presence of 0.3 M KCl. The final concentration of cobalt(II) in the samples was 1–2 mM as determined by measuring the absorption of the $Co(c)_2Zn(n)_2$–LADH at 350 nm. NAD^+ or NADH were added as solid powders to the solutions of the enzyme. Small amounts of a 0.5–1 M Tes or Taps solution (pH 9–10), containing 0.3 M KCl were added to the samples to change the pH. The pH was measured in the sample tubes with a microelectrode connected to a SEAC pH meter (Model S 106). The pH of the D_2O solutions is reported as uncorrected pH-meter reading (pH*).

The ^1H NMR spectra of the enzyme samples in H_2O or D_2O were run at 8 °C on a 60-MHz instrument based on a Bruker CXP 100 console equipped with 1.41 T Varian DA 60 electromagnet and an external lock circuit granting a ±1-Hz long-term stability. All spectra were recorded in quadrature detection mode on a 100-kHz spectral range. The strong signals of solvent protons and of all the diamagnetic protons of the protein have been suppressed by using the following modified DEFT pulse sequence $90°_x$–τ–$180°_x$–τ–$90°_x$–acquisition, which is also suited for performing T_1 measurements by recording spectra at different τ values.[17] The whole sequence was further phase alternated by using the standard Bruker PAPS sequence to reduce building up of coherent noise. The $90°_x$ and the $180°_x$ pulse lengths were adjusted by monitoring the water signal of each sample. Typical $90°_x$ pulse lengths were around 3 μs; typical τ values and recycle time values were around 30 and 45 ms, respectively. In order to obtain minimally distorted line shapes from the broadest signals in a wide spectral range the dead time between the last 90° pulse and the beginning of the signal acquisition was set to 10 μs. Spectra were accumulated for (1–6) × 10^5 scans, which, owing to the fast recycle time employed, required approximately from 3 to 18 h. Weighting factors of 50–100 Hz were applied. The sharper signal of the histidine 67 δ-NH proton was also recorded with weighting factors of 20 Hz. The chemical shifts have been measured from the H_2O or residual HDO signals and have been reported relative to Me_4Si by assuming a 4.8 ppm downfield shift for the H_2O signal. The shift value readings are accurate to ±0.4 ppm (equivalent to the digital resolution of the spectra), but the uncertainty is larger for the broadest signals. The 300-MHz ^1H NMR spectra were recorded on a CXP 300 spectrometer at Bruker Physik, Karlsruhe, F.R.G., by using spectral parameters similar to those discussed before.

The room-temperature electronic spectra in the UV, VIS, and near IR regions were run on a Cary 17D instrument. The absorption of the NMR samples in the visible region was routinely monitored before and after the NMR experiments to check for possible alterations of the enzyme. The CD spectra in the 700–250-nm wavelength region were recorded either on a JASCO 200-D or on a JASCO J-20 instrument.

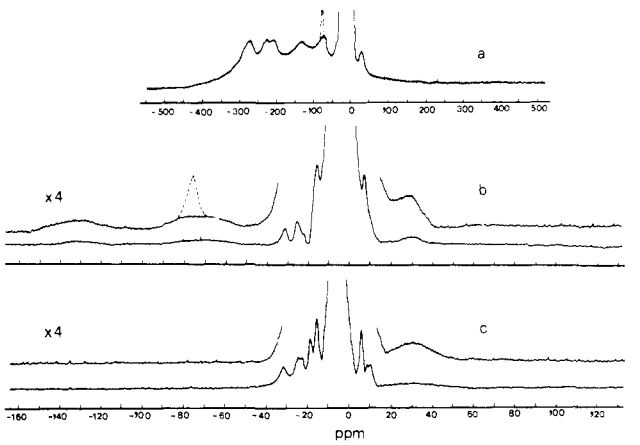
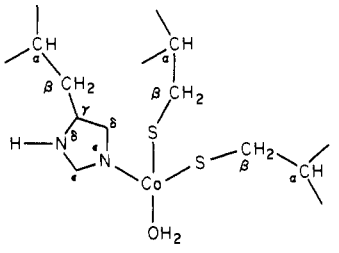


Figure 1. ^1H NMR spectra of $Co(c)_2Zn(n)_2$–LADH (33 mM Tes buffer pH 8.3, 0.3 M KCl) (a,b) 60-MHz spectra in D_2O; the dashed signal is present in H_2O solution. (c) 300-MHz spectrum in D_2O.

Results

$Co(c)_2Zn(n)_2$–LADH. The 60- and 300-MHz ^1H NMR spectra of $Co(c)_2Zn(n)_2$–LADH in D_2O solutions containing 33 mM Tes buffer at pH* 8.3 and 0.3 M KCl are shown in Figure 1. Five broad signals are present at −276, −226, −211, −131, and −72 ppm downfield from Me_4Si. Owing to the large isotropic shifts and to the large line widths, they have to be assigned to the protons close to the paramagnetic center. The only reasonable candidates for these signals are the four β-cysteine protons and the two histidine protons, δ′-CH and ε-CH (I).



I

The T_1 values of these signals measured in H_2O are around 1 ms, consistent with a strong interaction of the paramagnetic center as expected for protons adjacent to the donor atom. The small T_1 values do not allow us to distinguish between the various signals, because of instrumental limitations. Even the intensity is difficult to estimate due to the partial overlap of the signals. It can be tentatively proposed, also on the basis of the ^1H NMR spectra of the binary complexes (see below), that the signal at −276 ppm has intensity 2 and the other four have intensity 1. The lack of a sharper signal to be assigned to a δ′-CH group with respect to the coordinated histidine nitrogen is consistent with the histidine binding through ε′-nitrogen[14-16] as proposed from crystallographic data.[18] Histidine protons in cobalt(II)-substituted carbonic anhydrase and carboxypeptidase A are detected between −140 and −40 ppm.[14,15] Therefore the two least shifted signals are assigned to the δ′-CH and ε-CH protons from the metal-bound histidine. Several other signals of approximate intensity 1 are present on the tail of the residual diamagnetic signal from −33 to +30 ppm; in particular, signals at −33, −27, and +30 ppm can safely be considered isotropically shifted. For the former two, the T_1 values can be estimated around 10 ms and for the latter around 4 ms. Reasonable candidates are the two α-CH protons

(12) Zeppezauer, M. In "The Coordination Chemistry of Metalloenzymes"; Bertini, I., Drago, R. S., Luchinat, C., Eds.; Reidel: Dordrecht, 1983; pp 99–102.
(13) Dietrich, H.; Zeppezauer, M. *J. Inorg. Biochem.* **1982**, *17*, 227.
(14) Bertini, I.; Canti, G.; Luchinat, C.; Mani, F. *J. Am. Chem. Soc.* **1981**, *103*, 7784.
(15) Bertini, I.; Canti, G.; Luchinat, C. *J. Am. Chem. Soc.* **1982**, *104*, 4943.
(16) Bertini, I.; Lanini, G.; Luchinat, C. *J. Am. Chem. Soc.* **1983**, *105*, 5116.
(17) Hochmann, J.; Kellerhals, H. P. *J. Magn. Reson.* **1980**, *38*, 23.
(18) Eklund, H.; Nordström, B.; Zeppezauer, E.; Söderlund, G.; Ohlsson, I.; Boiwe, T.; Söderberg, B. O.; Tapia, O.; Brändén, C. I.; Åkeson, Å. *J. Mol. Biol.* **1976**, *102*, 27.

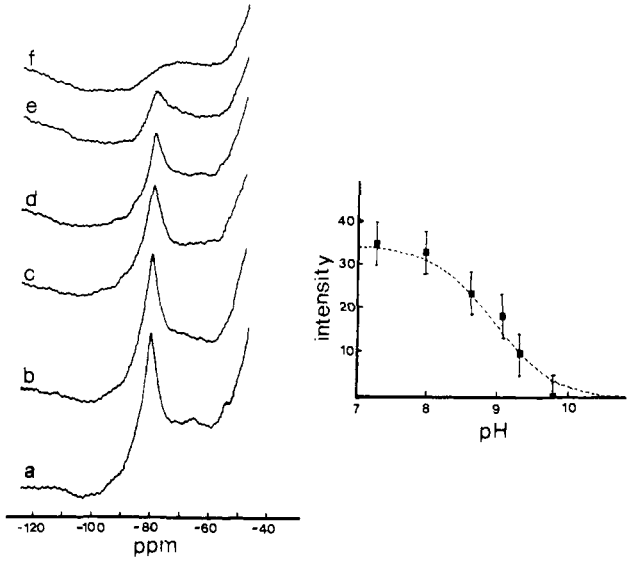


Figure 2. pH dependence of the His 67 δ-NH signal intensity: (a) pH 7.3, (b) pH 8.0, (c) pH 8.65, (d) pH 9.1, (e) pH 9.35, (f) pH 9.8. The data are best fitted to a pK_a of 9.0 ± 0.2.

of the cysteines and the two protons of the β-CH_2 of the histidine. The signals at −23, −18, and −16 ppm are relatively sharp signals that do not experience appreciable broadening upon changing the frequency from 60 to 300 MHz, whereas the former signals do, owing to Curie relaxation[19,20] (Figure 1). Therefore, the latter signals experience only a small, if any, paramagnetic effect and do not belong to the metal-coordinated residues.

The ^{1}H NMR spectra in H_2O show an additional signal at −80 ppm (Figure 1). On the basis of its chemical shift and line shape,[14,15] it has to be assigned to the δ-NH proton of histidine 67. Its T_1 value is 4.4 ms, consistent with other cobalt(II-substituted zinc enzymes.[16] At lower pH (6.3) the ^{1}H NMR spectrum remains unchanged as compared to the spectrum at pH 8.3. At higher pH, the broad signals far downfield become closer giving rise to an ill-resolved set of signals. Simultaneously, the δ-NH signal at −80 ppm decreases in intensity (Figure 2) without apparent broadening. The signal is below detection at pH 9.8, and no new signal is detected in any other part of the spectrum. The intensity of the δ-NH signal at pH's between 7.3 and 9.8 has been followed with a total of six measurements. From these data a pK_a of 9.0 ± 0.2 has been estimated. This shows that the δ-NH group of histidine 67 is involved in the acid–base equilibrium characterized by this pK_a.

Complexes of $Co(c)_2Zn(n)_2$–LADH with Coenzymes. The binary complex $Co(c)_2Zn(n)_2$–LADH/NAD^+ shows a ^{1}H NMR spectrum in the far downfield region comparable to that of $Co(c)_2Zn(n)_2$–LADH although of slightly lower quality (Figure 3). Therefore, the assignment of the signals is the same. The spectrum is pH dependent as well: the δ-NH signal shifts without decreasing in intensity from −63.4 ppm at pH 7.35 to −57.3 ppm at pH 9.2 with a pK_a of 8.3 ± 0.2 (see inset of Figure 3). The line width of this signal is essentially pH independent indicating that the interconversion between the acidic and basic forms is fast on the NMR time scale; i.e., the interconversion rate is larger than 1600 s^{-1}. The broad β-CH_2 signals of the cysteines are located at −279 and −223 ppm in the low-pH form and −222 and −208 ppm in the high-pH form. In the intermediate-pH range these signals become ill resolved as a result of eigher overlapping of the two different spectra or broadening caused by chemical exchange.

The behavior of the δ-NH signal of histidine 67 has also been followed at pH 8.4 starting from $Co(c)_2Zn(n)_2$–LADH and adding NAD^+ in less than stoichiometric amounts. Upon addition of NAD^+ the signal of the enzyme broadens at the beginning and then disappears, and finally the signal of the binary complex shows

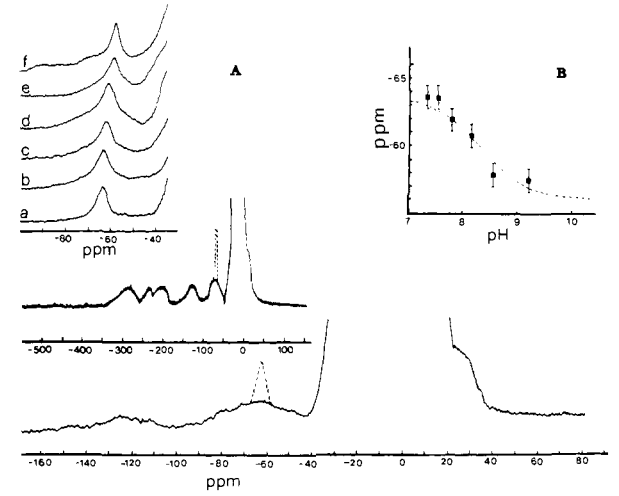


Figure 3. 60-MHz ^{1}H NMR spectrum of the binary complex with NAD^+ at pH* 7.35 in D_2O (conditions as in figure 1). The cobalt(II):NAD^+ ratio is 1:10. The dashed signal is the His 67 δ-NH signal present in H_2O solution. Inset A represents the ^{1}H NMR spectra at pH (a) 7.35, (b) 7.55, (c) 7.8, (d) 8.17, (e) 8.55, and (f) 9.2. Inset B represents the chemical shift of the δ-NH signal as a function of pH. The data are best fitted to a pK_a of 8.3 ± 0.2.

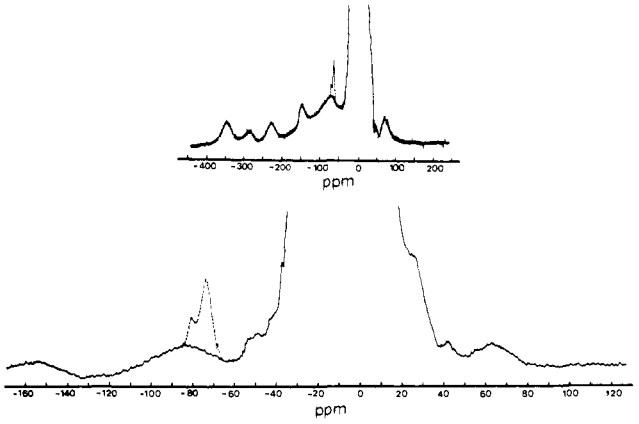


Figure 4. 60-MHz ^{1}H NMR spectrum of the binary complex with NADH at pH* 7.3 in D_2O (conditions as in Figure 1). The cobalt(II):NADH ratio is 1:2.5. The dashed signal at −80 ppm is the His 67 δ-NH signal of the residual pure enzyme form and that at −75 ppm is the His 67 δ-NH signal of the binary complex, which are present in H_2O solution.

up. The shift difference between the two positions is 20 ppm. If the exchange rate were $>10^4$ s^{-1} a single average NH signal would be expected under any condition, and if it were $<10^3$ s^{-1}, two signals would also be expected. The coalescence of the signal suggests that the coenzyme exchange rate is between these two limiting values. The data indicate that the exchange rate of NAD^+ in $Co(c)_2Zn(n)_2$–LADH is substantially lower than that reported for the native enzyme from kinetic data.[5] The sample containing NAD^+ apparently did not contain amounts of NADH detectable through electronic or NMR spectroscopy. This is in contrast to observations usually made with other LADH derivatives.[21]

The spectrum of the binary complex of $Co(c)_2Zn(n)_2$–LADH with NADH has been recorded at pH 7.3 and 9.3. The spectrum at pH 7.3 is shown in Figure 4. The signal intensities and line shapes correspond to those of $Co(c)_2Zn(n)_2$–LADH although the pramagnetic shifts are generally larger. The δ-NH signal is present at both pH values at the same position and with the same relative intensity. Therefore no acid–base equilibrium involving the active site of this binary complex was detected in this pH range. The presence in the spectrum of Figure 4 of significant amounts of

(19) Guéron, M. *J. Magn. Reson.* **1975**, *19*, 58.
(20) Vega, A. J.; Fiat, D. *Mol. Phys.* **1976**, *31*, 347.
(21) Eklund, H.; Plapp, B. V.; Samama, J.-P.; Brändén, C.-I. *J. Biol. Chem.* **1982**, *257*, 14349.

Table I. Near-Infrared Absorption Spectral Data of $Co(c)_2Zn(n)_2$-Horse Liver Alcohol Dehydrogenase and Its Binary Complexes with NAD^+ and NADH[b]

species	$10^{-3}\bar{\nu}$, cm^{-1}	ϵ, M^{-1} cm^{-1}
E (pH 6.2)[a]	10.0	90
E (pH 9.5)	10.0	140
E + NAD^+ (pH 6.2)	9.1	65
E + NAD^+ (pH 9.5)	9.5	65
E + NADH (pH 9)	11.1	70

[a] E = $Co(c)_2Zn(n)_2$-LADH. [b] The buffers used were 0.1 M Mes/K^+ at pH 6.2 and 0.1 M glycine/K^+ at pH 9.5. The spectra were run on a Cary 17D spectrophotometer at 21 °C.

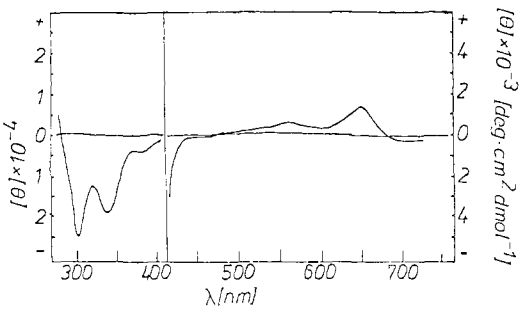


Figure 5. CD spectrum of $Co(c)_{1.1}Zn(n)_2$-LADH in 33 mM Tes/Na^+ at pH 6.9.

free enzyme may indicate that the affinity constant of cobalt(II) LADH for NADH is sizeably smaller in 0.3 M KCl than that of native LADH at smaller ionic strength.[22]

When NAD^+ and NADH are simultaneously present in such a ratio that both binary complexes are present, both δ-NH signals are present. This means that the coenzyme exchange rate is slow on the NMR time scale. The upper limit for the slower process is estimated to be around 3×10^3 s^{-1}.

The Electronic Absorption and CD Spectra. The electronic absorption spectra of all derivatives have been recorded in the range 8000–25000 cm^{-1} in order to check the integrity of the samples. The spectra in the visible region are identical with those reported earlier.[10,13,23] In the near-infrared region an additional band is observed in all the derivatives investigated (Table I). The CD spectrum of $Co(c)_2Zn(n)_2$-LADH is reported in Figure 5.

Discussion

The ^1H NMR signals of the β-cysteine protons and of the histidine protons are all downfield and are only slightly dependent on pH and type of derivative; this indicates that the nature of the isotropic shift is essentially contact in origin. The nonequivalence of the two protons of each β-CH_2 group of the cysteines is due to their rigidity as observed in ferredoxins.[24] The ^1H NMR spectra are consistent with the optical spectra in that no major change occurs in the coordination sphere of the catalytic metal ion upon binary complex formation with either oxidized or reduced coenzyme.

The crystallographic analysis of Zn_4-LADH and its ternary complexes with inhibitors have shown that the catalytic metal ion is tetracoordinate.[25] However, it has been recently proposed that the coordination number is increased to five upon binary complex formation with NAD^+.[26] The shape and intensity of the electronic absorption spectra of $Co(c)_2Zn(n)_2$-LADH extended down to the near-infrared region are consistent with a tetrahedral structure of the catalytic metal ion. In addition, there are no major spectral changes observed for the binary complexes in comparison with the unliganded enzyme (Table I). Formation of five-coordinate adducts would have caused a decrease in the intensity of the absorption spectrum[27] and a decrease in the nuclear relaxation rates.[16,28-30] Since a tetracoordinate structure for cobalt(II) has been established in $Co(c)_2Zn(n)_2$-LADH by X-ray crystallography,[2] the absence of spectral changes is strong evidence in favor of the retention of tetracoordinate structures in the observable complexes along the catalytic pathway.

A most remarkable result of this investigation is the disappearance of the δ-NH signal of the coordinated histidine 67 in the $Co(c)_2Zn(n)_2$-LADH with increasing pH. The pK_a of 9.0 for the disappearance of the NH signal can be determined only roughly because intensity measurements of different spectra are affected by large errors. However, since the signal is not detected anymore at pH 9.8, the pK_a can be estimated to be in the range 8.8–9.2. From electronic spectra of the $Co(c)_2Zn(n)_2$-LADH a pK_a had been located between 9.2 and 9.6 under slightly different conditions (23 °C, 0.1 M NaCl, 0.1 M buffer).[13] It has been postulated that this pK_a corresponds to the pK_a of 9.2 from kinetic data of Zn_4-LADH. It is reasonable to assume that the pK_a values detected by ^1H NMR and by electronic spectra are due to the same group. This group can possibly be the δ-NH of the metal-coordinated histidine; in this case the pK_a of the NH group would be substantially lower than 14.2, i.e., that of the free imidazole group, as expected for coordinated imidazoles.[31] Alternatively, another acid–base group may be responsible for the disappearance of the δ-NH proton signal at high pH; in this case, in order to acount for the signal disappearance without broadening, the acid–base equilibrium has to be slow on the NMR time scale and the basic species has to allow the δ-NH proton to exchange with bulk solvent rapidly. The two possibilities are summarized below:

$$E\text{-}His^*H^+ + HOH \overset{slow}{\rightleftharpoons} E\text{-}His\ H^+ + {}^*HOH \overset{pK_a\ 9;\ slow}{\rightleftharpoons} E\text{-}His + H_3O^+ \quad (A)$$

$$H^+\text{-}E\text{-}His^*H^+ + HOH \overset{slow}{\rightleftharpoons} H^+\text{-}E\text{-}His\ H^+ + {}^*HOH$$

$$pK_a\ 9;\ slow;\ -H^+ \quad (B)$$

$$E\text{-}His^*H^+ + HOH \overset{fast}{\rightleftharpoons} E\text{-}His\ H^+ + {}^*HOH$$

Unfortunately, from the analysis of the ^1H NMR spectra it is not possible to provide support to either one of the suggested possibilities. Undoubtedly NH deprotonation would be a quite tempting hypothesis.

In contrast to the behavior of the $Co(c)_2Zn(n)_2$-LADH, the δ-NH signal of histidine 67 in the binary complex with NAD^+ is present in the entire pH range investigated. Its pH-dependent shift has allowed us to locate the pK_a at 8.3 ± 0.2, which is in the same region as a pK_a detected through electronic spectroscopy.[13] Again, it has been postulated that this pK_a corresponds to the pK_a of 7.6 obtained from kinetic data on the native enzyme. In this case, of course, the ionizing moiety cannot be the histidine 67 NH group, and another acid–base group has to be looked for. Besides the histidine nitrogen, the only other metal ligand capable of deprotonation at the above pK_a is the coordinated water molecule. A water molecule coordinated to a tetrahedral cobalt(II) ion may have a pK_a as low as 6, for example, in bovine carbonic anhydrase B.[32] However, in horse liver alcohol dehydrogenase

(22) Iweibo, I.; Weiner, H. *Biochemistry* **1972**, *11*, 1003.
(23) Maret, W. Dissertation, Universität des Saarlandes, Saarbrücken, 1980.
(24) Holm, R. H.; Ibers, J. A. In "Iron Sulfur Proteins"; Lovenberg, W., Ed.; Academic Press: New York, 1977; Vol. 3, Chapter 7.
(25) Eklund, H.; Samama, J. P.; Wallén, L.; Brändén, C. I.; Åkeson, Å.; Jones, T. A. *J. Mol. Biol.* **1981**, *146*, 561.
(26) Makinen, M. W.; Yim, M. B. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6221.
(27) Bertini, I.; Canti, G.; Luchinat, C.; Scozzafava, A. *J. Am. Chem. Soc.* **1978**, *100*, 4873.
(28) Bertini, I.; Canti, G.; Luchinat, C. *Inorg. Chim. Acta* **1981**, *56*, 99.
(29) Bertini, I.; Brown, R. D.; Koenig, S. H.; Luchinat, C. *Biophys. J.* **1983**, *41*, 179.
(30) Bertini, I.; Luchinat C. In "Metal Ions in Biological Systems"; Sigel, H., Ed.; Marcel Dekker: New York, 1983; Vol. 15, pp 101–156.
(31) Hanania, G. I. H.; Irvine, D. H. *J. Chem. Soc.* **1964**, 5694. Hanania, G. I. H.; Irvine, D. H.; Irvine M. V. *J. Chem. Soc. A* **1966**, 296. Appleton, D. W.; Sarkar, B. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 1686.

the charge density at the catalytic metal ion is probably lower than in carbonic anhydrase due to its coordination to two negative, soft ligands, i.e., the thiolate groups of cysteines 46 and 174. Therefore, the pK_a is expected to be higher, and the observed value of 8.3 could be consistent with such a picture.

According to Petterson's and Shore's proposals,[9,33] the pK_a's observed in the native, unligated enzyme (pK_a = 9.2) and in the NAD^+ adduct (pK_a = 7.6) are both assigned to the coordinated water molecule, the shift in pK_a being due to the interaction of water with the positive nicotinamide moiety of NAD^+.[1] If this is the case, in light of the present ^1H NMR data the water proton dissociation in the $Co(c)_2Zn(n)_2$-LADH would have to be slow on the NMR time scale, as discussed before. Cook and Cleland[34] have suggested that in the binary complex with NAD^+ histidinium 51 could deprotonate instead of or simultaneously to the coordinated water. This hypothesis cannot be ruled out although it would hardly affect the isotropic shifts of the protons of coordinated residues.

The present data could be consistent with the ionizing group in the NAD^+ adduct being the coordinated water and in the $Co(c)_2Zn(n)_2$-LADH the histidine 67 δ-NH. As already stated, both the δ-NH of the coordinated histidine and the coordinated water may have pK_a values in the above region. Once one of the two groups has undergone deprotonation, the other is expected to shift several units in pK_a toward higher values. Another possibility is that deprotonation of amino acid side chains, even far from the active site, may cause a rearrangement of ligands in the active site. Such conformational changes have been reported.[35] As a result the δ-NH of the coordinated histidine would undergo a pH-dependent shift in the NAD^+ adduct and would exchange rapidly in the high-pH form of $Co(c)_2Zn(n)_2$-LADH. Such a hypothesis would be consistent (even if there are other possibilities) with the finding that the NADH association rate with $H_4Zn(n)_2$-LADH, i.e., the enzyme depleted of its catalytic zinc ion, shows a pH dependence with pK_a of about 8.8 and the NAD^+ dissociation rate from the binary complex of $H_4Zn(n)_2$-LADH with NAD^+ is still pH dependent, with a pK_a similar to that observed with the native enzyme.[8] Therefore the involvement of the metal-bound water in the acid-base equilibria that control coenzyme binding is now less likely.

The reported data require a reevaluation of current ideas about the mechanism of horse liver alcohol dehydrogenase. None of the proposed mechanisms[4] considers the involvement of histidine 67 in the catalytic cycle. The conclusion drawn from $Co(c)_2Zn(n)_2$-LADH are also valid for the native zinc enzyme. Schneider et al. have shown that the coordination structure of the catalytic metal ion is largely preserved in $Co(c)_2Zn(n)_2$-LADH.[2] Only a small tilt of histidine 67 has been detected. This rotation may cause a small perturbation of the pK_a of the δ-NH of histidine 67. Future studies will focus on the question whether and to what extent histidine 67 participates in the acid-base equilibria in LADH. If it turns out to be involved, the entire proton-transfer pathway from the metal-bound alcohol to the protein surface will have to be reconsidered.

Acknowledgment. This work was partially supported by Deutsche Forschunsgemeinschaft, Fonds der Chem. Industrie, Bundesminister für Forschung und Technologie, and by the Italian Secretariat for Public Education. Thanks are due to Bruker AG, Karlsruhe, for the use of the 300-MHz CXP spectrometer.

(32) Bertini, I.; Luchinat, C.; Scozzafava, A. *Inorg. Chim. Acta* **1980**, *46*, 85.
(33) Evans, S. A.; Shore, J. D. *J. Biol. Chem.* **1980**, 255, 1509.
(34) Cook, P. F.; Cleland, W. W. *Biochemistry* **1981**, *20*, 1805.
(35) Wolfe, J. K.; Weidig, C.; Halvorson, H. R.; Shore, J. D.; Parker, D.; Holbrook, J. J. *J. Biol. Chem.* **1977**, *252*, 333.

Locating Paramagnetically Shifted ^{13}C NMR Resonances in Cobalt-Substituted Carbonic Anhydrase. A Reverse Inhibitor Titration Method

Pamela Jeffers Morley,[1a,b] Steven B. Carroll,[1a] Patricia Harmon,[1c] and Raja G. Khalifah*[1c-e]

Contribution from the Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901, and Biochemistry Department, University of Kansas Medical School, and Veterans Administration Medical Center, Kansas City, Missouri 64128. *Received June 27, 1983*

Abstract: Human carbonic anhydrase (EC 4.2.1.1) isozyme I (formerly HCAB), a zinc metalloenzyme, contains an active-site histidine-200 that is specifically reactive with bromoacetate. We previously used 90% [1-^{13}C]bromoacetate to modify the enzyme and extensively studied the enriched carboxylate by using ^{13}C NMR. These diamagnetic studies suggested that under specific pH conditions the carboxymethyl carboxylate acts as an *intramolecular* ligand of the catalytically essential zinc, thus possibly accounting for the changes in catalytic activity that result from the modification (Jeffers, P. K.; Sutherland, W. M.; Khalifah, R. G. *Biochemistry* **1978**, *17*, 1305). Studies on the cobalt-substituted enzyme, which is nearly equally active to the zinc enzyme, were attempted in order to measure metal-carboxylate distances to verify this mechanistically important hypothesis. However, the carboxylate resonance could only be detected in the presence of inhibitors that were known to bind at the zinc. Presumably, the external inhibitors were displacing the intramolecular carboxylate ligand, thus reducing possibly large paramagnetic perturbations that render it undetectable. It is shown here that when azide is used as the displacing inhibitor, fast-exchange conditions appear to prevail, suggesting that a "reverse titration" with azide could lead to an extrapolation of the chemical shift to the uninhibited position in absence of azide. The validity of this procedure has been confirmed directly by searching for and locating the paramagnetically shifted resonance in absence of azide using maximum spectral sweep widths at pH 8.7 and 50.32 MHz. The large carboxylate line width of 1100 Hz and its chemical shift at 207 ppm at this pH account for its unobservability in earlier attempts using more limited instrumentation. This method may prove equally useful in predicting the relaxation properties of the paramagnetically shifted resonance when its direct observation is still not possible. It may also have applicability in studies on other metalloenzyme systems.

The observation, assignment, and utilization of paramagnetically perturbed resonances in NMR can be quite challenging due to the characteristically large line widths and chemical shift perturbations that may result. However, the study of such resonances

0002-7863/84/1506-1830$01.50/0