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.

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

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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

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can be potentially extremely useful due to the wealth of molecular information that can be extracted from them.[2] In the case of coordination compounds, NMR observation is often limited to resonances of ligands that are in fast exchange between free and coordinated environments, such that only a small fraction is coordinated. Suitable extrapolation of concentration-dependent parameters can then be employed to deduce the properties of the paramagnetic complex of interest. Paramagnetic relaxation studies on substrates and inhibitors of metalloproteins and metalloenzymes using this approach have been pioneered by Mildvan[3] and others[4,5] and have provided a wealth of useful geometric, structural, and mechanistic information. This approach is not possible, however, when the resonances of interest are those of intrinsic or extrinsic covalently attached groups in a paramagnetic metalloenzyme.

We wish to describe a novel method applicable to the study of intramolecular ligands of paramagnetic metalloenzymes that permits the determination of the properties of paramagnetically shifted ligand resonances. Since it is not possible to carry out concentration dependence studies on covalently attached ligands, we have devised a competition experiment in which the fraction of intramolecular ligand coordinated is modulated by titrating the enzyme with an external inhibitor that displaces it from the metal. The method has been used with cobalt-substituted carbonic anhydrase (EC 4.2.1.1) isozyme I from humans, formerly designated as HCAB, where the resonance of interest is a ^{13}C-enriched carboxylate anchored close to the metal by carboxymethylation at histidine-200 of the active site. Our previous studies on the diamagnetic zinc form deduced a possible coordination of the carboxylate to the metal under certain pH conditions,[6] but attempts to verify this by metal distance measurements on the cobalt-substituted enzyme were prevented by the unobservability of the paramagnetically shifted ^{13}C resonance in this metallo-derivative. The success of the method has been demonstrated by direct observation of the shifted resonance in the *uninhibited* enzyme at the location predicted by the "reverse inhibitor titration" method.

Experimental Procedures

Enzymatic Procedures. Human carbonic anhydrase isozyme I was prepared by affinity chromatography[7] from freshly outdated erythrocytes. It was carboxymethylated at His-200 with 90% [1-^{13}C]bromoacetate (KOR Isotopes) according to established procedures.[7] Intrinsic zinc was removed by dialysis against pyridine-2,6-dicarboxylic acid[8] and was replaced by the addition of a stoichiometric equivalent of cobalt sulfate (Specpure grade of Johnson-Matthey). The determination of the inhibition constant for azide was carried out by using *p*-nitrophenylacetate esterase activity assays.[9] Inhibition constants were determined by three-parameter nonlinear regression analysis.

NMR Spectroscopy. Low-field measurements at 25.15 MHz were carried out on a JEOL PFT/EC 100 Fourier transform spectrometer using a 10-mm probe at an ambient temperature of 298 K. A 5-kHz crystal filter limited maximum sweep widths to 5 kHz. Measurements at 50.32 MHz were carried out on a Bruker WP-200 Fourier transform spectrometer using quadrature detection and a maximal sweep width of 50 kHz. Ambient probe temperature with broad-band proton decoupling was 304 K.

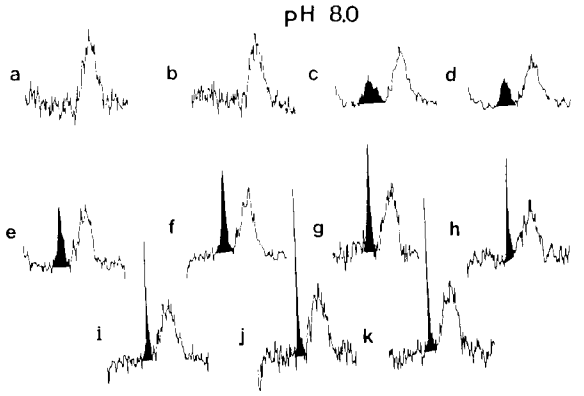

Figure 1. Effect of azide inhibition at pH 8.0 on the 25.15-MHz ^{13}C NMR spectrum of cobalt-substituted carbonic anhydrase I carboxymethylated at His-200 using 90%-enriched [1-^{13}C]bromoacetate. The downfield carbonyl carbon region is shown (roughly from 160–200 ppm). The shaded resonances belong to the enriched carboxyl carbon while the broad peak is the natural abundance envelope of the protein carbonyl and carboxyl carbons. In (a–k) azide concentration was increased as follows (mM units): 0.5, 1.0, 2.5, 3.0, 4.0, 4.6, 5.6, 10.5, 20.1, 62.9, and 98.7, respectively. Initial enzyme concentration was 3.04 mM and final was 2.45 mM.

Results and Discussion

The enriched carboxylate of carboxymethylated carbonic anhydrase I (ZnCmCA I) exhibits an easily detected resonance in the 172–176 ppm (downfield from $(CH_3)_4Si$) range that is very sensitive to changes in pH and the addition of inhibitors to the active site,[6,7,10-13] as expected from the proximity of the modified His-200 to the essential zinc. Replacement of the Zn(II) by the paramagnetic Co(II) ($S = {}^3/_2$) rendered the resonance undetectable at the field of our initial studies (25.15 MHz, 5-kHz maximum sweep widths) at all the pH range of roughly 5.5–10.5 that is accessible.[6] However, we subsequently discovered that inhibition of Co^{II}CmCA I with inhibitors known to bind as anions at the active-site zinc led to the reappearance of the resonance. These complexes showed moderate shifts from the diamagnetic positions (<10 ppm) and narrow line width.[13] Presumably the pseudocontact effects that account for these changes are much less than in the uninihibited enzyme due to an increase in the carboxylate distance from the metal resulting from competition between the carboxylate and inhibitors for binding to the metal.[11,14] These observations suggested that examining the spectra of CoCmCA I in the presence of decreasing concentrations of a suitable inhibitor that is in *fast exchange* may lead to progressive changes that can be extrapolated to zero inhibitor concentration. Such an extrapolation would yield the desired chemical shift or relaxation parameters of the *uninhibited* CoCmCA I enzyme.

Figure 1 shows that the well-known inhibitor azide appears to fulfill the requirements. Decreasing concentrations of azide lead to progressive downfield shifts and line-width increases until the resonance is too broad to detect. The chemical shift of the uninhibited CoCmCA I (δ_E) was then estimated by assuming fast-exchange conditions,

$$\delta_{obsd} = f_{EI}\delta_{EI} + f_E\delta_E = ([EI]/E_0)\delta_{EI} + ([E]_{free}/E_0)\delta_E \quad (1)$$

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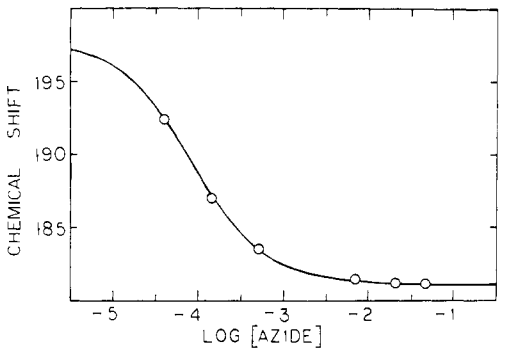

Figure 2. Concentration dependence of the enriched carboxylate resonance as a function of the computed free azide concentration for an inhibitor titration experiment at pH 8.8 similar to that shown in Figure 1. The curve through the points was obtained by three-parameter nonlinear regression analysis, which gave a pK_i of 4.1 ± 0.1 (compared to 3.3 determined by assay) and limiting chemical shifts of 181.3 ± 0.1 ppm for δ_{EI} and 197.8 ± 0.2 ppm for δ_E. The probable error of the latter limiting shift is likely to be much larger than the stated precision, since the regression did not take into account the increase in uncertainty of the shifts as the resonance became very broad at low inhibitor concentrations.

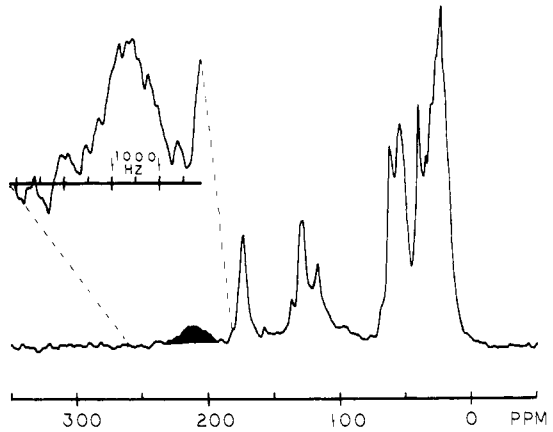

Figure 3. 50.32-MHz ^{13}C NMR spectrum of cobalt-substituted carbonic anhydrase I carboxymethylated at His-200 with 90%-enriched [1-^{13}C]-bromoacetate taken on a Bruker WP-200 spectrometer operating in quadrature detection mode and using 50-kHz sweep width. Spectrum is average of 105 000 scans. The enriched carboxylate resonance near 200 ppm is indicated by the shaded area and was verified by performing an azide titration experiment similar to that in Figure 1. Inset shows an expansion of this resonance to indicate its approximate line width. The pH was 8.7.

where E_0 is the total enzyme concentration and δ_E and δ_{EI} are the carboxylate chemical shift in CoCmCA I and its azide complex, respectively. The inhibition of the cobalt-substituted carboxymethylated enzyme by azide was assumed to be simple, so that the fractions of enzyme free and inhibited were obtained at any azide concentration using

$$K_i = ([E]_{free}[I]_{free})/[EI] \quad (2)$$

where the K_i value was determined independently by an enzymatic assay or was determined from the fitting of the chemical shift data. The concentration of the chemical shift was then analyzed by two-parameter or three-parameter nonlinear regression analysis to yield the limiting values δ_{EI} and the long-sought δ_E, as shown in Figure 2 for data obtained at pH 8.8.

Direct verification of the validity of this method has been obtained under conditions where the paramagnetic shift is predicted to be moderate (about 30 ppm). Figure 3 demonstrates the successful observation of the carboxylate of CoCmCA I at 50.32 MHz using an instrument equipped with quadrature detection and 50-kHz sweep width. The resonance is seen to be extremely broad, having an approximate line width of 1100 Hz. It occurs in the approximate region predicted by analysis of previous azide titration experiments (Figure 2), the small differences being probably due to the uncertainties in the extrapolation of the latter type of experiment. Approximate fast-exchange behavior of this resonance has been demonstrated by an azide titration study (not shown) where it was progressively followed until the enzyme was completely inhibited. Its spectral characteristics readily account for its unobservability under the previous instrumental conditions.

The direct observation of the paramagnetically shifted resonance now opens the way for *direct* spin–lattice relaxation studies that should lead to an estimate of the distance between the carboxylate and the metal. Such studies are in progress and should lead to an eventual verification of the zinc–carboxylate hypothesis[7] mentioned above that is of much mechanistic significance. Our preliminary results to date indicate, however, that the method we have described above for deducing the properties of the uninhibited CoCmCA I will be indispensable in these studies, since the carboxylate undergoes much larger chemical shift changes and broadening at other pH ranges that render it undetectable. Indirect studies by this reverse inhibitor titration method are thus being utilized under such conditions. Our approach should be useful in the study of other metalloenzymes and metalloproteins. It may also have applications where a fast-exchange ligand may be found to induce diamagnetism or decreased paramagnetism in other systems, such as the heme proteins.

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Registry No. Carbonic anhydrase, 9001-03-0; cobalt, 7440-48-4; azide, 14343-69-2; [1-^{13}C]bromoacetic acid, 57858-24-9.