^{17}O NMR Studies of Carbonic Anhydrase

K. D. Rose† and R. G. Bryant*

Contribution from the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received February 20, 1979*

Abstract: ^{17}O NMR relaxation rates are investigated in aqueous solutions of carbonic anhydrase and several metal derivatives. The ^{17}O NMR relaxation rates are dominated by protein-induced modulations of the proton lifetime on solvent oxygen atoms. Direct effects of the water molecules possibly coordinated to the active site metals were observed only in the case of the manganous derivative. It is shown that interpretation of ^{17}O NMR data must carefully take proton lifetimes into account as they are sensitive to subtle changes in the protein conformation. In particular it is found that a significant change in the solvent proton lifetime attends reconstitution of the apoenzyme with Zn(II), Cd(II), and Co(II) at neutral pH values.

Introduction

Metal ion function in metalloenzyme active sites has been of interest for many years.[1] A basic problem in focusing on the indigenous metal atom in the enzyme structure is that the molar concentration of the metal is very low, thus affording poor signal to noise for many direct spectroscopic approaches. One strategy to circumvent this problem involves substitution of the indigenous metal with one having more favorable spectroscopic properties.[2] This technique suffers from the obvious fact that the structural and dynamic properties of the modified enzyme are not necessarily the same as those of the native enzyme. Indeed, the reaction mechanism of a metalloenzyme may well vary between different substrates even when the catalytic metal is not changed. Several NMR relaxation methods exploit properties of nuclei that exchange with the metal ion first coordination sphere or with nuclei that are bonded to atoms in the metal first coordination sphere. Examples of these include chloride ion[3,4] and protons bonded to oxygen in the first coordination sphere of the metal.[5] Even in these cases interpretation of the relaxation data is uncertain with respect to defining the details of the water molecule structure in the active site region either because water protons rather than water molecules are measured or the characteristics of water in the native active site region must be inferred from data on other ions and molecules.

It is of considerable interest to understand the structural and dynamic properties of water in the active sites of metalloenzymes since subtle but cooperative effects involving water may severely affect the catalytic events. There is evidence from halide ion NMR relaxation measurements that there are important interactions between the first coordination sphere of the metal and other residues in the active site regions of carboxypeptidase A[6,7] and perhaps also carbonic anhydrase.[8] In addition it has been suggested that water may provide the crucial link between the metal chemistry and the non-first coordination sphere ligands in the active site vicinity; however, it is difficult to study these subtle interactions directly by most spectroscopic techniques. The present NMR investigation of ^{17}O was motivated by the desire to determine whether the first coordination sphere interactions of active site metals can be explored by this technique without the inherent complications of less direct methods.

It has not been definitively resolved whether there are any water molecules tightly associated with the protein molecule in a protein solution. Evidence from NMR relaxation measurements suggests that, while there may be a large number of perturbed water molecules in the vicinity of the protein, the interactions are short lived and do not involve tight sticking of the water to the protein.[9] This suggestion is supported by studies of the solvent on the protein surface in relatively dry systems where motion is also found to be very rapid.[10] Nevertheless, if a water molecule were to be bound to the protein surface in a way that eliminated rotation of the water molecule with respect to the protein, then relatively large effects should be seen in the solvent NMR relaxation.[11] The first coordination sphere of a metal in a metalloenzyme would seem to provide an almost optimal choice for a water molecule that is intimately associated with the protein and may satisfy these motional criteria as well. Rotational freedom of metal-coordinated water is possible even in solids;[12] however, in the metalloenzyme active site region a hydrogen bond from a metal-coordinated water molecule to an adjacent functional group may limit the rotational correlation time of the water molecule to that of the protein. In this favorable case, and if exchange with the bulk solvent occurs rapidly on the time scale of the relaxation time of the water molecule in the metal coordination sphere, then observation of the bulk resonance will reflect this specific active site interaction. With these conditions the NMR relaxation equation becomes[13]

$$\Delta\nu_{\text{total}} = P_{\text{f}}\Delta\nu_{\text{f}} + P_{\text{Zn}}\Delta\nu_{\text{Zn}} + P_{\text{E}}\Delta\nu_{\text{E}} \qquad (1)$$

where P_{f}, P_{Zn}, and P_{E} are the probabilities that the oxygen atom observed is located in the bulk of the solution, at the zinc first coordination sphere, or at any of several other possible sites on the protein that are as yet unspecified and perhaps unknown. Neglecting the asymmetry parameter and non-extreme-narrowing effects,[14] the line widths, $\Delta\nu$, in eq 1 will each be given approximately by

$$\Delta\nu = \frac{12\pi}{125}\left(\frac{e^2qQ}{h}\right)^2 \tau_c \qquad (2)$$

where eQ is the nuclear electric quadrupole moment and τ_c describes the correlation time for the reorientation of the electric field gradient, eq, at the observed oxygen nucleus.[15] Appropriate control experiments permit isolation of the zinc-atom contribution to the total relaxation equation. Assuming that the water molecule correlation time in the bulk solvent is on the order of picoseconds and that for the protein-bound water molecule on the order of 10 ns, the change in the correlation time for water sensed by the ^{17}O nucleus upon binding to the first coordination sphere of the metal in the active site is optimistically estimated to be on the order of 10^4. If the changes in the quadrupole coupling constant in eq 2 are small when water coordinates to the metal, then, based on the correlation time change alone, the line width for the oxygen atom at the zinc site in the protein will be 10^4 times that in the water molecule free in solution or about 5×10^5 Hz. With an enzyme concentration of 2 mM, P_{Zn} becomes 3×10^{-5} and the zinc site contribution to eq 1 becomes approximately 15 Hz. Such a change in the relaxation rate in the oxygen resonance may be easily resolved on our spectrometer, suggesting that ^{17}O

† Exxon Research and Engineering, P.O. Box 121, Linden, N.J. 07036.
* Camille and Henry Dreyfus Teacher-Scholar.

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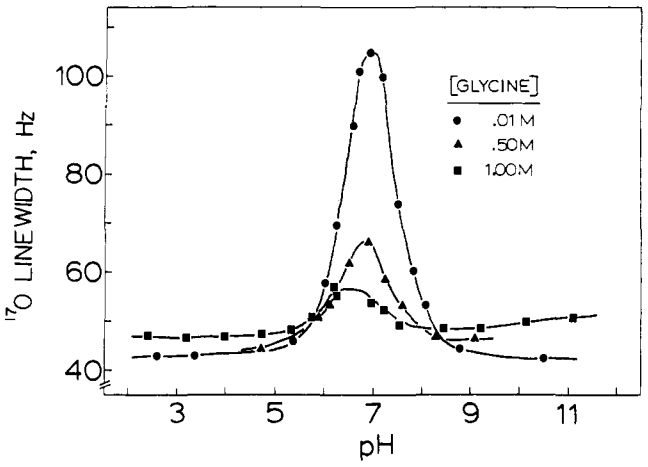

Figure 1. ^{17}O NMR line width recorded at 8.1 MHz and 303 K for aqueous solutions of glycine at several concentrations as a function of pH.

NMR relaxation may provide a direct method for investigating the subtleties of the water active site chemistry even in diamagnetic metalloenzymes. It is important to note that, since this effect is a consequence of a change in the quadrupole relaxation contribution, the predicted line-width enhancement should be observable in the longitudinal and transverse relaxation rates as well as in the presence of other line-broadening mechanisms. For these experiments, we have chosen to investigate bovine carbonic anhydrase because it is well characterized by a wide variety of methods including NMR relaxation and is stable at high concentration in aqueous solutions. The metal-free or apoenzyme and metal-substituted enzymes are also reasonably stable and easy to prepare and have been extensively investigated by activity and inhibition studies. Although the high enzyme concentrations are often a problem, carbonic anhydrase has the advantage over the proteolytic zinc enzymes that it does not self-destruct.

Experimental Section

^{17}O NMR relaxation rates were measured at 8.1 MHz in a Varian 12-in. electromagnet which was field frequency locked to an external hexafluoroacetone sample at 56.4 MHz for ^{19}F. The spectrometer utilizes a Nicolet NMR-80 data system for pulse sequencing, data acquisition, and analysis, a Hewlett-Packard 8660B synthesized signal generator for the oxygen frequency source, and ENI broad-band amplifiers for pulse amplification. The receiver was constructed in this laboratory.[16] The single coil probes made in this laboratory required 90° pulse widths of 45 μs from 10-W amplifiers. Broad-band proton decoupling was accomplished by phase modulation of the proton frequency using a saddle coil probe configuration. Calibration of the proton decoupling efficiency was confirmed on a neutral pH enriched ^{17}O sample prior to each series of decoupling experiments. Proton-coupled spectra were accumulated under identical conditions except that the ^{1}H frequency was raised by 1 MHz. The sample temperature was maintained at 303 K. Transverse relaxation rates for ^{17}O were determined either from the line width of the Fourier transformed free induction decay or by an amplitude weighted least-squares analysis of the on-resonance free induction decay.[16] Individual samples required 0.8% $^{17}OH_2$ enrichment and 2-min acquisition times.

Carbonic anhydrase from bovine erthyrocytes was obtained from Sigma. The esterase activity was determined using *p*-nitrophenyl acetate (Sigma) as substrate by the method of Armstrong et al. as described in the Miles-Servac catalog.[17] No differences in enzyme activity or ^{17}O relaxation measurements were observed within 5% between native and Zn^{2+} reconstituted enzyme samples. Enzyme concentrations were determined spectrophotometrically assuming an extinction coefficient at 280 nm of 5.7×10^4 M^{-1} cm^{-1} and mol wt of 31 000[18] or by dry weight.

The metal-free or apoenzyme was prepared according to Hunt et al., with the exception that buffers were avoided throughout the demetalization procedures.[19] Apoenzyme samples and acetazolamide (twofold) inhibited BCA gave identical activities and were not greater than 2% residual activity compared to native enzyme run concurrently.

$^{17}OH_2$ (21.7%) (Norsk Hydro) used for sample enrichment was stored over Chelex-100 to avoid introduction of adventitious metal ion contaminants into apoenzyme solutions. All other reagents were purchased commercially and used without further purification. Distilled deionized water was used throughout.

Results and Discussion

The ^{17}O NMR relaxation data for aqueous glycine solutions in Figure 1 demonstrate the well-known fact that the oxygen transverse relaxation rate in water is pH dependent. This observation was exploited by Meiboom to extract the rate constants for acid- and base-catalyzed proton exchange in water.[20] The source of this effect is classified by Abragam as a scalar interaction of the first kind; that is, the scalar coupling between the proton and oxygen magnetic moments is modulated by a chemical-exchange event, in this case the proton exchange from one oxygen to another. As shown in Figure 1, the addition of a proton-exchange catalyst, such as glycine, chemically decouples the protons from the oxygen nuclei by decreasing the proton lifetime. A protein represents a potentially efficient proton-exchange catalyst because it provides a number of solvent-accessible acidic or basic functional groups. To separate the contributions to ^{17}O relaxation of the metal site from other protein interactions, as previously suggested, care must be taken to monitor changes, if any, in the proton lifetime contribution to the solvent ^{17}O relaxation rate. After trying several chemical control experiments for such a differentiation, we find that the simplest reproducible way to eliminate exchange effects when desired is to proton decouple while observing the oxygen resonance. This technique selectively discriminates between the proton-dependent ^{17}O relaxation mechanisms and all other contributions.[21]

^{17}O NMR relaxation data are shown in Figure 2 as a function of pH for reconstituted and apocarbonic anhydrase solutions. Also shown are oxygen relaxation rates obtained for the same solutions with the proton spins decoupled from the oxygen. The data in Figure 2 demonstrate that a concentrated metalloenzyme solution preserves the significant pH dependence of the ^{17}O transverse relaxation rate, as observed for dilute glycine solutions (Figure 1). In contrast the ^{17}O relaxation rate near neutral pH in the apoenzyme solution is significantly smaller, demonstrating that there is a ^{17}O relaxation contribution which depends on the presence of zinc in the enzyme structure. The data in Figure 3 further show that the relaxation rate enhancement at neutral pH may be quantitatively recovered by the addition of 1 equiv of zinc ion to the apoenzyme. Similar results were obtained with the cadmium and cobalt reconstitutions of the apoenzyme.[22]

Although the difference between zinc reconstituted and apoenzyme ^{17}O proton-coupled relaxation rates is certainly associated with the presence of zinc in the active site structure, it is not possible to associate this difference with one or more immobilized water molecules in the zinc ion first coordination sphere. In fact, relaxation rate enhancements from nuclear electric quadrupole interactions are not observed between these samples since, as seen in Figures 2 and 3, proton decoupling yields identical ^{17}O NMR relaxation rates for the apo- and reconstituted enzyme solutions. Furthermore, the addition of potent inhibitors of carbonic anhydrase such as acetazolamide (Figure 3), sulfide, or cyanide ions does not change the differences observed between the reconstituted and apoenzyme solutions. Finally, in view of the proton decoupling results, if the relaxation rate difference between the reconstituted and apoenzyme solutions is ascribed to the zinc site alone, the calculated magnitude of the proton-dependent relaxation contribution to the ^{17}O from such a specific interaction is far

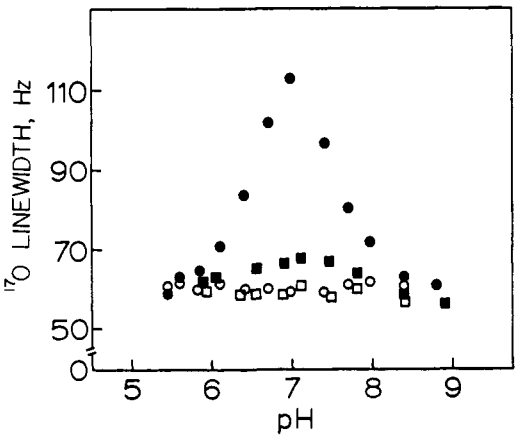

Figure 2. ^{17}O NMR line widths recorded at 8.1 MHz and 303 K for reconstituted and apocarbonic anhydrase solutions at 0.65 mM as a function of pH at an oxygen enrichment of 0.5%: ●, reconstituted enzyme; ○, proton-decoupled reconstituted enzyme; ■, apoenzyme; □, proton-decoupled apoenzyme.

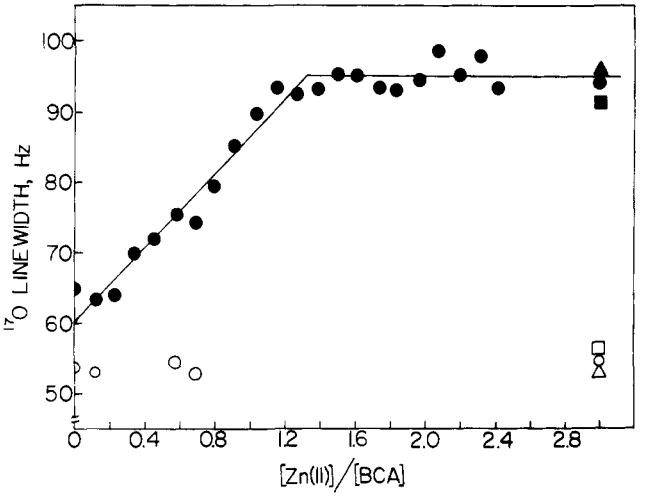

Figure 3. ^{17}O NMR line width recorded at 8.1 MHz, 303 K, and pH 7.1 for a 0.84 mM bovine carbonic anhydrase apoenzyme as a function of the zinc(II) ion to enzyme ratio. Closed and open symbols refer to proton-coupled and -decoupled measurements, respectively. At 3.0 equiv the sample was remeasured before (▲,△) and after (■,□) addition of 3 equiv of acetazolamide inhibitor.

too large to be accounted for by either an unusual oxygen–proton scalar interaction of the second kind or by a direct dipole–dipole mechanism.[23] These results suggest that, although the ^{17}O relaxation rate depends on the reconstitution of the apoenzyme, the relaxation contribution is not a consequence of a zinc site specific interaction of water. This is consistent with earlier conclusions based on proton relaxation data that the interaction of water with a protein is dynamically subtle and not easily explained by postulating a significant number of irrotationally bound water molecules.[24]

The lack of significant line broadening or pH dependence for the proton-decoupled ^{17}O spectra of the enzyme solutions may result from several sources: (1) Water may not bind to the zinc atom directly. (2) Water may be displaced from the zinc atom in the solutions studied by another ligand such as chloride ion[25-29] so that the metal contribution to the ^{17}O relaxation is eliminated. (3) A zinc-coordinated water molecule does not exchange sufficiently fast to provide rapid exchange averaging of the relaxation rates appearing in eq 1. (4) The relaxation rate for a water molecule in the zinc site of the protein is smaller than predicted.

The weight of zinc chemistry as well as the results of other probe experiments on the zinc enzyme suggests that it is unlikely that the zinc ion is normally three coordinate in the enzyme. It also appears unlikely that a nonwater ligand completely eliminates water coordination in the present experiments since proton-coupled line-width measurements are the same for Zn^{2+} reconstituted enzyme samples prepared in the absence of salt, in 0.1 M NaCl, and in 0.1 M $NaNO_3$.

Two possibilities would cause an overestimate of the zinc ion contribution to the ^{17}O line width in the enzyme solution. In the absence of hydrogen bonding between first coordination sphere water and neighboring active site residues, a water molecule coordinated to the active site zinc ion may rotate relative to the enzyme molecule as a whole. This rotation would effectively dilute the correlation time change experienced by the water on binding to the metal site and hence make the effect too small to observe at practical enzyme concentrations.[29-31] A second alternative hinges on the assumption that the electric field gradient appearing in eq 2 is constant when the water coordinates to the zinc; it is quite possible that a decrease in the field-gradient results from the metal–water coordination which partially compensates the large correlation time change anticipated on binding. To test this possibility the ^{1}H decoupled ^{17}O NMR line width was measured in the presence of 0.4 M hexaaquozinc(II) ion and found to be 45 Hz, while that of the control was 40 Hz. This difference is roughly that expected for the change in solution viscosity alone so that the line width of the oxygen coordinated to the zinc ion must be similar to that in the uncoordinated water. Since the hexaaquozinc(II) ion rotates more slowly than an uncoordinated water molecule by about a factor of 3,[32] it appears that the field gradient experienced by the oxygen in the zinc first coordination sphere may be smaller than in the uncoordinated water if effects of water-molecule rotation in the complex may be neglected. While field-gradient changes on coordination of the oxygen may be small,[31] a significant decrease in the quadrupole coupling constant may not be ruled out as a cause for the absence of a metal-induced broadening in the enzyme solution.

Regardless of the reason for the lack of a zinc-ion contribution in the ^{17}O NMR relaxation experiments, we are unable to draw any conclusions about the nature of the presumed zinc-coordinated water molecule or to state anything definitive about its acid–base properties based on these measurements. Since proton decoupling eliminates all proton-dependent contributions to the oxygen relaxation rate and since a change in the bulk water proton exchange rate, as in the glycine data of Figure 1, is the only proton mechanism that appears consistent with all the experimental results, the difference between the native and apoenzyme solution data may be ascribed to a change in the proton-exchange rate among oxygen atoms in the solvent. This conclusion has been qualitatively confirmed by observation of the proton spin-echo dispersion for native apoenzyme samples enriched in ^{17}O using the Carr–Purcell–Meiboom–Gill spin-echo pulse sequence.[16,33-35] Thus the observed change in the ^{17}O proton coupled line widths is a consequence of changes in the proton-exchange lifetime which in turn depends on the state of reconstitution of the carbonic anhydrase apoenzyme.

Quantitative consideration of the ^{17}O line-width data shows that in spite of the large number of potential proton-exchange catalysts that the high concentration of protein brings to the solution, the proton lifetime decreases by only 30% when the protein is dissolved. Removal of zinc ion further decreases the proton-exchange lifetime by approximately a factor of 4. The only chemical change attending removal of zinc is the exposure of three histidine residues to the solvent; however, independent measurements on imidazole solutions at pH 7.2 indicate that at least 80 mM imidazole is required to achieve the same decrease in proton-exchange lifetime, i.e., more than 30 times the

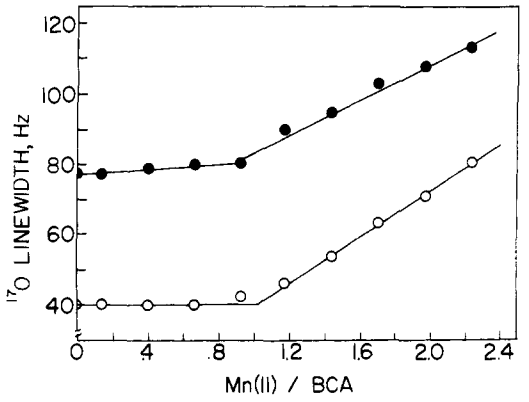

Figure 4. ^{17}O NMR line width recorded at 8.1 MHz, 303 K, and pH 7.1 for a 0.084 mM bovine carbonic anhydrase apoenzyme solution as a function of the metal to enzyme ratio. Closed and open symbols refer to proton-coupled and -decoupled measurements, respectively.

change in imidazole concentration achieved by zinc ion removal from carbonic anhydrase. Therefore we suggest that the solvent interactions of a significant number of protein residues change upon zinc ion removal, presumably as a result of a small protein conformational change. This conclusion is consistent with all the experimental results but is difficult at this time to model since the number and types of exposed residues are not known and the exchange rate constants for many of these residues have not been reported.

Though a protein conformational change is apparently observed by ^{17}O NMR, there is little evidence from other experimental techniques that a substantial conformational adjustment occurs in bovine carbonic anhydrase by removal of the active-site zinc. For example there is no difference in the sedimentation coefficients between the zinc and the metal-free enzymes,[36] but there is a very small change suggested by fluorescence depolarization measurements.[37] Small changes in tritium exchange,[38,39] fluorescein fluorescence,[37] UV absorption,[38] and ORD spectra[38,40] are also reported on addition of the zinc ion to apoenzyme solutions which have been interpreted to indicate a modest rearrangement of the enzyme structure.[40] The present ^{17}O data, which are apparently more sensitive to the enzyme conformational state than these other techniques, suggest that the nature of the conformation change attending metal removal is a minor unfolding of the carbonic anhydrase molecule that brings several groups capable of catalyzing proton exchange into more intimate contact with the solvent.

Reconstitution of the apoenzyme with a paramagnetic metal such as manganese(II) ion yields different results as shown in Figure 4, where both proton coupled and decoupled ^{17}O NMR are reported. The apoenzyme concentration in this case is more dilute by a factor of 10 than in the previous experiments; therefore the effects of enzyme reconstitution demonstrated in Figure 3 will be negligible since these scale as the protein concentration. The data of Figure 4 show a dramatic increase in the proton-coupled and decoupled line widths when the ratio of metal to enzyme exceeds unity. In this case the effect of paramagnetic interactions on the ^{17}O relaxation is small as long as the paramagnetic ion is bound in the active site region of the enzyme. This observation may result from the enzyme sequestering all metal ion first coordination sphere positions or more simply a decrease in the number of available coordination sites compared to free solution metal ion. This distinction may not be confirmed from the data of Figure 4. However, ^{17}O data collected at higher enzyme concentrations[22] and a wealth of ^{1}H measurements on manganous carbonic anhydrase strongly suggest that the additional break point in Figure 4 is a consequence of the fewer number of metal ion coordination positions in the enzyme case. Although further interpretation will not be undertaken at this time, it is clear that the ^{17}O NMR relaxation of water does provide a direct method for monitoring the extent of metal ion binding to the apoenzyme.

In summary we have shown that the major effects on the ^{17}O NMR relaxation rate in diamagnetic protein solutions are caused by changes in the proton exchange rate among solvent molecules on addition of the protein and that any analysis by difference spectroscopy must take very careful account of these changes before concluding anything about the nature of the protein–water interaction.[42] The most direct method for eliminating this complication in ^{17}O NMR measurements is to take advantage of proton-decoupling techniques. It appears from these results that ^{17}O NMR may be usefully exploited as a very sensitive monitor of protein conformation.

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