PREPRINT UCRL- 82332

Lawrence Livermore Laboratory³⁵Cl and ⁸¹Br Nuclear Magnetic Resonance Studies of Carbonic Anhydrase

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February 1979

This paper was prepared for presentation at the Symposium on Biophysics and Physiology of Carbon Dioxide, Regensburg, West Germany, April 17-20, 1979.

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Studies of Carbonic Anhydrase

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Introduction

Mammalian erythrocytes often contain more than one type of carbonic anhydrase and human erythrocytes in particular yield two principal forms of the enzyme, which are distinguished foremost by their relative specific activities in regards to the catalysis of the important reversible carbon dioxide hydration reaction. Although these isozymes exhibit many other differences, they have in common the stringent requirement of one zinc ion per molecular weight unit of about 30,000.

The binding of a single monovalent anion by carbonic anhydrase is sufficient to abolish the hydrolysis of CO₂ by the enzyme.⁽¹⁾ A model often proposed for this inhibition of carbonic anhydrase is competitive binding between the monovalent anion and water molecules for a zinc coordination site. ³⁵Cl nmr studies have substantiated the binding of Cl⁻ to the Zn(II) of carbonic anhydrase and have proved useful in studies of the properties of the active site Zn(II) of a number of the mammalian enzymes.

The nuclear relaxation properties of Cl⁻ (nuclear spin of 3/2) are determined by the interaction of the nuclear quadrupole moment with time varying electric fields experienced by the nucleus. Aqueous chloride ions are symmetrically solvated by water molecules and because of vanishingly small electric fields at the nucleus exhibit moderately narrow nmr absorption line widths of ~ 10 Hz. Distortion of the electric field at the nucleus, for example, by covalent bonding, yields broad nmr absorption line widths in the kHz range.

If chloride ions are capable of being in more than one type of environment with no exchange between these environments, the nmr spectrum will consist of a superposition of peaks each indicative of the particular environment. If the chloride ions exchange among these sites rapidly compared to the relaxation time in a particular site, then an averaged nmr absorption will be observed. In the

particular case of interest here, carbonic anhydrase is present in small quantities, 10^{-4} - 10^{-5} M, compared to aqueous chloride ions (~ 0.5 M). The nmr experiment consists in observing the effect of the carbonic anhydrase-chloride interaction on the more readily detectable 0.5 M Cl^- absorption.

In detail, the line width of the ^{35}Cl resonance, as measured by the full width at half-maximum amplitude, is related to the relaxation time of the nucleus by the expression $\pi\Delta\nu = 1/T_2$. When a moderately fast chemical exchange of the chloride ion occurs between a free solvated environment and another environment in which the nucleus has an intrinsically different relaxation time, the observed relaxation time will be the weighted average of the relaxation time characteristic of the two environments. (2) Thus, if $1/T_2(0)$ represents the relaxation rate of the free chloride ion and $1/T_2$ the observed relaxation rate, then the relaxation due to the bound chloride ion is given by:

$$1/T_{2P} = 1/T_2 - 1/T_2(0) \quad (1)$$

When no chemical shift results from the different environment experienced by the chloride ion on binding

$$\frac{1}{T_{2P}} = f/(T_{2M} + \tau_M) \quad (2)$$

where T_{2M} is the transverse relaxation time of the protein bound chloride ion, τ_M is the exchange lifetime, and f is the fraction of the total chloride ion which is bound to the protein.

The relaxation mechanism for the ^{35}Cl nucleus in the absence of paramagnetic species is the interaction between the nuclear quadrupole moment of the nucleus (eq) and the fluctuating electrostatic field gradient (eq) determined by the environment and sensed at the nucleus. The full nmr line width for a particular environment is given by

$$\Delta\nu_1 = \frac{2}{3} \pi (e^2 q_1 Q) g(t_c) \quad (3)$$

$$\text{where } g(t_c) = 0.6 t_c + \frac{t_c}{1 + \omega^2 t_c^2} + \frac{0.4 t_c}{1 + 4\omega^2 t_c^2} \quad (4)$$

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where t_c is the characteristic correlation time of the nucleus in that environment and ω is the nuclear precessional frequency.

It is important to distinguish between exchange limited and rapid exchange cases and this can be done by examining the temperature dependence of the line width⁽³⁾ or by comparing the ratio of the ^{35}Cl and ^{37}Cl line widths. For a nucleus which relaxes by a quadrupole related process, the rate is proportional to the correlation time for molecular tumbling. This rate decreases with increasing temperature and thus the line width would be expected to decrease with increasing temperature for the rapid exchange situation. By contrast, τ_M invariably decreases with increasing temperature and from equation (2) a larger line width will result with increasing temperature. In the fast exchange situation equation 2 reduces to

$$\frac{1}{T_{2P}} = \frac{f}{\sigma_{2m}} \quad (5)$$

while in the exchange limited case the equation reduces to

$$\frac{1}{T_{2P}} = \frac{f}{\tau_M} \quad (6)$$

For carbonic anhydrase, as will be shown, the exchange limited case pertains.

Results

The nmr line width of aqueous solutions of Cl^- up to about 1 molar is of the order of 10 Hz. Addition of Zn(II) in the mM concentration range to aqueous Cl^- produces an increase in the Cl^- line width. This increase varies linearly with the Zn(II) concentration and can be calculated from the expression

$$\Delta\nu_{\text{obs}} - \Delta\nu_{\text{Cl}^-} = 2 \times 10^3 [\text{Zn(II)}] \quad (7)$$

If the Cl^- nmr line width is responsive to aqueous Zn(II) with its correspondingly small t_c , carbonic anhydrase which possesses a molecular correlation time a thousand times larger should be even more effective in broadening the Cl^- resonance.

The first isozyme of carbonic anhydrase studied by the ^{35}Cl nmr technique was the bovine enzyme (BCA).⁴ In fact, the first experiment was carried out with such a large amount of the enzyme, 25 mg/ml, that the Cl^- resonance was broadened beyond detection. Subsequent studies revealed that carbonic anhydrase is approximately a thousand times more effective in broadening Cl^- than aqueous Zn(II) . Furthermore, while the broadening produced by aqueous Zn(II) is pH independent up to the point where Zn(II) begins to precipitate, the Cl^- broadening produced by CA exhibits a strong pH dependence. A pH titration of the Cl^- line width for a solution of bovine CA is illustrated in Figure 1. This titration curve illustrates a common feature of all CA isozymes where the Cl^- broadening is large at low pH (6-7) and decreases in a sigmoidal manner as the pH is increased. In addition, the midpoint of the pH curve in 0.5 M Cl^- can be correlated with the type of isozyme, i.e., a high or low activity form of the enzyme. All high activity forms, i.e., BCA, human carbonic anhydrase C (HCAC) and horse CA-C have a lower pH midpoint than the low activity forms (HCA-B, horse-CA-B), Figures 2, 3, 4 and 5. The magnitude of the Cl^- broadening is a function of the individual enzyme and is pretty much independent of preparation (assuming a reasonably purified enzyme). There is some correlation of maximum broadening with activity classification, Table 1, but this correlation has not been extensively examined.⁵

Lindskog⁶ and Lindskog and Thorslund⁷ studied the effect of anions on the pKa of the group controlling the activity of carbonic anhydrase. Their data showed that anions that bind more tightly increase the pKa more than less inhibitory species. We have observed that the pKa values obtained from the pH variation of Cl^- line width also varies with the Cl^- concentration. An analysis of the Cl^- dependence in conjunction with kinetically determined inhibitor constants⁸ (K_i of 2 mM for HCA-B and 200 mM at pH 6.5 for HCA-C) yields on extrapolation to zero chloride a pKa value of 7.0 for HCAB and 6.7 for HCAC. These values are in close agreement whereas the difference observed in 0.5 M Cl^- is 1.8 units. Differences in pKa values are magnified as the Cl^- concentration increases and the values divide into two groups which correspond to the high activity form of the enzyme (low pKa) and the low activity form of the enzyme (high pKa).

The Cl^- broadening produced by CA has been attributed to the binding of Cl^- to the single Zn(II) ion located at the bottom of the active site crevice described in the crystallographic studies.^{9,10} For both the human enzymes the Zn(II) ion is bound to three histidine residues thereby leaving a formal +2 zinc ion with one coordination site available for a water molecule or Cl^- . The actual Zn(II) environment is one of a slightly distorted tetrahedral symmetry.

In order to substantiate the claim of a $\text{Zn(II)}-\text{Cl}^-$ interaction, we have prepared Zn(II) free carbonic anhydrase and observed its effect on the Cl^- line width. ApoCA exhibits essentially no effect on the Cl^- line width when compared to the native enzyme. When the apoenzyme is titrated with aqueous Zn(II) ions and monitored by the Cl^- line width, the line width increases linearly until a maximum is reached at a mole ratio of one Zn(II) to one CA. The line width levels off after a 1:1 ratio has been reached and remains essentially constant as the Zn(II) concentration increases.

Cyanide inhibits the enzymatic activity of all carbonic anhydrases. A CN^- titration of native BCA is shown in Fig. 6 and demonstrates competition of Cl^- for the CN^- binding site. One concludes from this titration that there is only one available $\text{Zn(II)}-\text{Cl}^-$ binding site. Acetazolamide, a potent sulfonamide inhibitor of CA, also affects the Cl^- line width and completely removes the Cl^- broadening at a mole ratio of 1:1. Crystallographic studies reveal that it is the anion form of the sulfonamide which binds to the enzyme within the coordination site of the Zn(II) ion. Co(II) substituted carbonic anhydrase possesses properties quite similar to the native Zn(II) enzyme.¹¹ Co(II) CA broadens the Cl^- nmr line width in a manner similar to that of the native Zn(II) enzyme. Figure 8 is a titration of apobovine CA with μl amounts of CoCl_2 . The Cl^- broadening reaches a maximum at a 1:1 stoichiometry of Co(II) to BCA which agrees with titrations of apocarbonic anhydrase with Zn(II) . Similarly, Co(II) in excess of 1:1 has little effect on the Cl^- line width in agreement with the Zn(II) results. A pH titration of Co(II) BCA and Co(II) HCA-B appears in Figure 9. Like the native enzyme, the high and low activity forms of the Co(II) enzyme exhibit a difference in the pKa's measured in 0.5 M Cl with the low activity form possessing the higher pKa.

Smith and Bryant¹² have examined the effect of Hg(II) and Cd(II) substituted carbonic anhydrase on the Cl^- line width and observed that the line width was not broadened. This is quite interesting since crystallographic studies reveal an octahedral Hg(II) coordinated to four amino acids and two Cl^- ions. As indicated later, in the discussion of ^{81}Br nmr studies, this is undoubtedly a result of slow exchange of bound Cl^- .

Mechanism of Chloride Ion Relaxation

Equation 2 relates the increase in line width of Cl^- in the presence of CA to the inverse sum of two terms, T_{2M} and τ_M . T_{2M} is a measure of the effect of the electric field gradient experienced by Zn(II) bound Cl^- and the molecular tumbling of the molecule. τ_M is the lifetime of Cl^- bound to the Zn(II). As indicated, a temperature study can sort out these effects. Figure 10 displays the effect of temperature on the net line width of HCAB at pH 6.5 and 8.6. Since the net Cl^- line width increases with temperature, the lifetime of Zn(II) bound Cl^- , τ_M , is the dominating factor in the Cl^- relaxation. The value of k_{off} for the reaction

$\text{Zn(II)}-\text{Cl} \xrightleftharpoons[k_{\text{on}}]{k_{\text{off}}} \text{Zn(II)} + \text{Cl}^-$ at 25°C is $1. \times 10^6 \text{ sec}^{-1}$ and $7.3 \times 10^5 \text{ sec}^{-1}$ at pH 6.5

and 8.6 respectively.³ Additional evidence that τ_M determines the relaxation time was derived from comparative $^{35}\text{Cl}/^{37}\text{Cl}$ line width measurements. If the relaxation is controlled by T_{2M} of equation 2, then the ratio of the $^{35}\text{Cl}/^{37}\text{Cl}$ line widths would be in the ratio of the quadrupole moments squared or 1.61. If τ_M , i.e., the exchange rate, determines the relaxation rate, then the ratio of the $^{35}\text{Cl}/^{37}\text{Cl}$ line widths should be 1.0. We measured this ratio at 14.09 kG and found it to be 1.0 substantiating that the lifetime of Cl^- bound to the Zn(II) of carbonic anhydrase dominates the relaxation behavior.

^{81}Br nmr behavior is quite similar to ^{35}Cl in that its relaxation is dominated by quadrupolar interactions. ^{81}Br line widths are ~45 times larger than ^{35}Cl primarily because of the larger quadrupole moment. We have examined high (BCA) and low activity (HCAB) forms of carbonic anhydrase using ^{81}Br nmr and observed a striking difference in the effect of the two forms on the ^{81}Br line width.

BCA produces an effect on the ^{81}Br line width that is in all aspects quite similar to the results reported for the ^{35}Cl studies. HCAB, however, produces a nonspecific broadening of the ^{81}Br line width that is not responsive to either

CN⁻, acetazolamide, or pH, Fig. 11. Competitive Br⁻/Cl⁻ experiments using ³⁵Cl nmr indicate that Br⁻ competes with Cl⁻ for the Zn(II) binding site. We, therefore, conclude that the lifetime of Br⁻ bound to be Zn(II) ion of HCAB is much longer than for BCA and the slowness of the exchange prevents an observable increase in the aqueous Br⁻ line width. Interestingly, a chemically modified form of HCAB, which has an uncharged carboxyamidomethyl group attached to His-200 located near the active site, does exhibit ⁸¹Br nmr results similar to the ³⁵Cl studies, Fig. 12.

Acid Titration Results

Some of the pH titration curves, Figures 2, 4 and 5 exhibit a decrease in line width as the pH is decreased below neutrality. A comparison of this effect with uv difference spectral measurements has been made for HCAC and HCAB dissolved in 0.15 M Cl⁻ solutions, Figures 13 and 14. The Cl⁻ line width measurements in the acid region are different for the two enzymes. The Cl⁻ measurements for the C enzyme closely parallel those of Riddiford.¹⁴ The small displacement in the curves may be due to differences in temperature, 32.9° vs room temperature. The sharp change in the Cl⁻ line width at pH 5 for the C enzyme is irreversible. If the pH of one solution is changed from 6 to 4.9 the line width decreases to a minimum value and remains narrow as the pH is raised again to ~6, where the protein precipitates. The Cl⁻ line widths as a function of pH for the B enzyme, in the acid region, do not parallel Riddiford's curve. The decrease in Cl⁻ broadening with decreasing pH is much more gradual and is reversible. At pH 4.1, however, the protein begins to come out of solution. It is in this pH region that HCAB undergoes an irreversible conformational change.¹⁴ Although the reason for the decrease in Cl⁻ line width as the pH is decreased is not known, it is known that in the pH region, 5-7, the Zn(II) remains firmly bound to the protein and therefore the decrease in Cl⁻ line width cannot be attributed to removal of Zn(II).

Acknowledgements

I would like to thank my associates and coworkers who have made much of this work possible and, in particular, Jim Happe, Al Maddux, and Bert Holder. I would also particularly like to express my thanks to Professor John T. Edsall whose encouragement and supplies of the human and horse enzymes made this work both possible and exciting.

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Table I: Measured pK for Isozymes of Carbonic Anhydrase in 0.5 M NaCl.^a

	pK	Molar Relaxivity (Hz/M)
I. Horse CA-B	9.85	5.7×10^5
Human CA-B	9.22	6×10^5
II. Human CA-C	7.42	1×10^6
Horse CA-C	7.23	5×10^5
Bovine CA-B	7.3	2×10^6

^aThe molar relaxivity is the maximum observed value in 0.5 M NaCl.

Figure Captions

- Figure 1. The increase in ^{35}Cl line width, $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz), vs. pH for a solution containing 0.5 M NaCl and 2.29×10^{-5} bovine carbonic anhydrase.
- Figure 2. $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz) vs. pH for a solution containing 0.62 mg/ml of human carbonic anhydrase C in 0.5 M NaCl. The pK value is 7.42.
- Figure 3. $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz) vs. pH for a solution containing 1.27 mg/ml of horse carbonic anhydrase C in 0.5 M NaCl. The pK value is 7.23.
- Figure 4. $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz) vs. pH for a solution containing 1.5 mg/ml of human carbonic anhydrase B in 0.5 M NaCl. The pK value is 9.22.
- Figure 5. $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz) vs. pH for a solution containing 1.28 mg/ml of horse carbonic anhydrase B in 0.5 M NaCl. The pK value is 9.85.
- Figure 6. $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz) vs. molar ratio of CN^-/BCA for a solution containing 0.5 M NaCl, 3.8×10^{-5} M bovine carbonic anhydrase, and 0.05 M Tris buffer (pH 8.2).
- Figure 7. $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz) vs. molar ratio of acetazolamide/BCA for a solution containing 0.5 M NaCl, 4.05×10^{-5} M bovine carbonic anhydrase, and 0.05 M Tris buffer (pH 8.05).
- Figure 8. Line width, $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz), vs. mole ratio $\text{Co}^{2+}/\text{apo bovine carbonic anhydrase}$. The solution was 0.5 M NaCl, 0.54 mg per ml of apo bovine carbonic anhydrase and 0.05 M Bis-Tris, pH 6.15.
- Figure 9. Line width, $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz) vs. pH for a solution of apo HCAB, 0.9 mg/ml, and 3.1×10^{-5} M Co(II) and a solution of apo BCA, 0.86 mg/ml, and 2.86×10^{-5} M Co(II) both in 0.5 MCl⁻.
- Figure 10. Arrhenius plot of net chloride line broadening for human carbonic anhydrase B (circles) pH 6.4 (1.5 mg/ml) and triangles) pH 8.59 (3 mg/ml) in 0.5 M NaCl. The f factor was calculated using a value of K_f of 0.2 M.
- Figure 11. Net line width, $\Delta\nu_{\text{net}}$, i.e., $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Br}^-}$, vs. pH for solutions of bovine carbonic anhydrase (solid symbols), 0.5 mg/ml, and human carbonic anhydrase B (open symbols), 4 mg/ml, in 0.5 M NaBr. The pKa for the bovine enzyme is 8.6.

Figure 12. Net line width, $\Delta\nu_{\text{net}}$, i.e., $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Br}^-}$, vs. pH for a solution of modified (carboxyamidomethyl) human carbonic anhydrase B, 3.4 mg/ml, in 0.5 M NaBr. The pKa is 9.6.

Figure 13. $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz) vs. pH for a solution containing 0.3 mg/ml of human carbonic anhydrase C in 0.15 M NaCl and $\Delta\epsilon$ (dashed curve) as a function of pH at 291.5 m μ in 0.15 M NaCl (redrawn from Riddiford, 1965).

Figure 14. $\Delta\nu_{\text{obsd}} - \Delta\nu_{\text{Cl}^-}$ (Hz) vs. pH for a solution containing 0.69 mg/ml of human carbonic anhydrase B in 0.15 M NaCl and $\Delta\epsilon$ (dashed curve) as a function of pH at 291.5 m μ in 0.15 M NaCl (redrawn from Riddiford, 1965). The diamonds are for a solution containing 0.53 mg/ml; the line width was normalized accordingly.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

If color is to be added

- Make a stat or film positive of the finished line art
- Add the color to the stat or positive and bleed any color background to this border

Figure 9

Size 50 %
 VU-graphs 50 %
 Prints 50 %

Figure 10

Figure 11

Figure 12

Figure 13

Figure 14