Zinc Environmental Differences in Carbonic Anhydrase Isozymes*

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ABSTRACT: The interaction of the Zn(II) metal ion with its environment and with chloride ions has been investigated by ^{35}Cl nuclear magnetic resonance spectroscopy for human B and C and horse B and C carbonic anhydrase. The Zn(II)–chloride interaction in these systems is the dominant relaxation mechanism for ^{35}Cl nuclei. The pH dependence of the ^{35}Cl relaxation rate clearly distinguishes between the Zn(II) ion in high and low specific activity forms of the enzyme. The observed pK depends on the total chloride concentration. Measurement of pK as a function of chloride concentration yields an extrapolated pK_h of 6.4 and 8.19 for the high (C) and low (B) specific activity forms of the human enzyme. A comparison study of human B and C, horse B and C, and the bovine enzyme suggests that all high-activity and low-activity forms of mammalian carbonic anhydrase can be separated into groups with pK_h's near these values. Acid titration studies of the human C enzyme in 0.15 M NaCl closely parallel the ultraviolet difference spectra of Riddiford (*J. Biol. Chem. 240*, 168 (1965)). The ^{35}Cl nuclear magnetic resonance data indicate that there is no change in the zinc environment until the protein begins to unfold at about pH 5. Acid titration studies of the human B enzyme in 0.15 M NaCl, however, reveal a reversible change in the zinc environment before the unfolding of the protein begins. Cyanide titration studies indicate one available zinc coordination site for all the isozymes studied. The binding of 1 equiv of acetazolamide/carbonic anhydrase reduces the chloride line width to that of the cyanide-inhibited enzyme.

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.

A previous report (Ward, 1969) has demonstrated that ^{35}Cl nuclear magnetic resonance line width or relaxation rate measurements can be used to investigate the properties of the zinc ion of bovine carbonic anhydrase. This article is primarily concerned with the ^{35}Cl nuclear magnetic resonance studies of the separated B and C forms of both the human and horse carbonic anhydrases.

These studies have demonstrated that the high specific activity form of the human (C) enzyme possesses an environment for the zinc ion such that its pK_h for hydrolysis is 6.4, while the low specific activity form of the human (B) enzyme has a zinc pK_h of 8.19. Acid titration studies of the human C enzyme closely parallel the ultraviolet difference spectra studies of Riddiford (1965) in that no effect on the zinc environment is observed by ^{35}Cl nuclear magnetic resonance studies until the protein begins to unfold. Similar studies with the human B enzyme reveal a change in the zinc environment before the unfolding of the protein begins. Cyanide and acetazolamide titrations of the human and horse enzymes are in agreement with those performed on the bovine enzyme in that the binding of one inhibitor per carbonic anhydrase molecule reduces nearly to zero the chloride broadening.

Experimental Section

Materials. Samples of human B and C and horse B and C carbonic anhydrases were a gift from Professor J. T. Edsall. Human B and C enzymes were also isolated in this laboratory from red blood cells kindly supplied by the Veterans' Hospital of Livermore. The procedure for the isolation was that outlined by Professor Edsall (personal communication). The horse enzymes were isolated by A. Furth. The horse B enzyme preparation contained a small amount of hemoglobin and was further purified by DEAE-Sephadex chromatography. The horse C enzyme preparation contained traces of minor C enzyme artifacts. Protein solutions were concentrated by dialysis against solid ammonium sulfate and stored in concentrated ammonium sulfate at 4°. All samples used in ^{35}Cl studies were dialyzed thoroughly against EDTA–Tris-chloride buffer (pH 8) and then Tris-chloride buffer or water. At no time were the solutions lyophilized. All pH measurements were made with a Corning Model 12 pH meter with a Beckman combination electrode standardized against common buffers.

Nuclear Magnetic Resonance Measurements. The ^{35}Cl spectra were obtained at 5.88 MHz with a Varian V-4311 fixed radiofrequency unit. A PAR Model HR 8 lock-in amplifier was used for field modulation and phase-sensitive detection. The spectrometer was equipped with a field-frequency lock system. The radiofrequency field level was low enough so that saturation effects were absent. The ^{35}Cl line widths were measured from recorded spectra as the full width at half-maximum amplitude. The reported line

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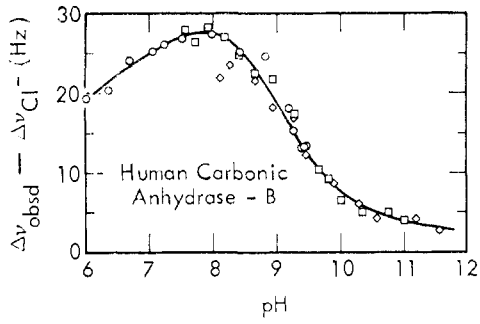

FIGURE 1: $\Delta\nu_{obsd} - \Delta\nu_{Cl^-}$ (Hz) *vs.* pH for a solution containing 1.5 mg/ml of human carbonic anhydrase B in 0.5 M NaCl. The p*K* value is 9.22.

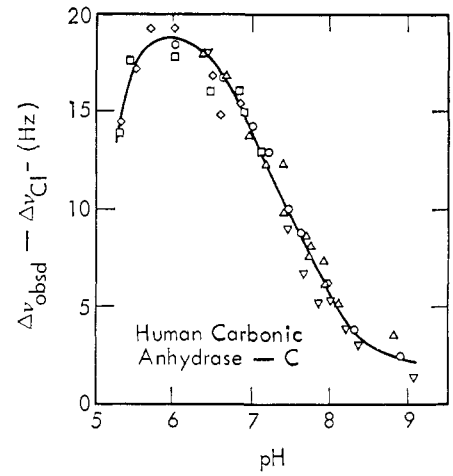

FIGURE 2: $\Delta\nu_{obsd} - \Delta\nu_{Cl^-}$ (Hz) *vs.* pH for a solution containing 0.62 mg/ml of human carbonic anhydrase C in 0.5 M NaCl. The p*K* value is 7.42.

widths are the average of usually eight to ten spectra and are reproducible to about 5%. The resolution was such that 0.5 M aqueous NaCl exhibited a line width, $\Delta\nu_{Cl^-}$, of 11.0 ± 1 Hz. Relaxation rate and line widths are related by the expression, $\pi\Delta\nu = 1/T_2$. All spectra were obtained at the probe temperature, 32.9°.

Theory. As noted earlier (Ward, 1969), interactions between the electric quadrupole moment and fluctuating electric field gradients at the nucleus provide the dominant nuclear magnetic relaxation mechanism for chlorine nuclei in solution. Aqueous chloride ions, however, exhibit a fairly narrow, 11.0 ± 1 Hz, ^{35}Cl nuclear magnetic resonance absorption because of symmetrical hydration by water molecules. For those environments in which chlorine nuclei experience electric field gradients, the line width is given by (Abragam and Pound, 1953)

$$\Delta\nu = \left(\frac{2\pi}{5}\right)[e^2qQ]^2\tau \qquad (1)$$

where $\Delta\nu$ is the full width at half-maximum amplitude, q the electric field gradient at the nucleus, Q the electric quadrupole moment of the nucleus, and τ is a correlation time which describes the random molecular motions responsible for the time-varying electric field gradients.

For those systems in which chloride ions can exist at various sites, such as bound to Zn(II) or as free aqueous chloride ions, and for which the exchange rate of the chloride ion among these sites is rapid compared with the width of the broadest line, a composite signal is observed. This composite signal can be described by the expression $\Delta\nu = \Sigma(\Delta\nu_i)P_i$, where P_i is the probability that chloride ions are at site i. All ^{35}Cl line widths are reported as the observed line width, $\Delta\nu_{obsd}$, minus the line width of aqueous 0.5 M NaCl, $\Delta\nu_{Cl^-}$. It should be noted that the ^{35}Cl line width of aqueous NaCl varies little in the concentration ranges considered here.

It has been shown previously (Ward, 1969) that the zinc–chloride interaction is the dominant cause of the increased relaxation rate of ^{35}Cl nuclei in the presence of carbonic anhydrase. The large increase in the relaxation rate over that due to aqueous zinc–chloride interactions is attributed to the much longer effective correlation time, $\sim 10^{-8}$ sec, compared with the shorter rotational correlation time, 10^{-11} sec, of the aquo zinc–chloride complex. It will therefore become apparent that changes in the observed chloride line widths can be interpreted in terms of either the accessibility of the zinc ion to chloride or changes in the molecular reorientation time of the enzyme.

Results

Carbonic Anhydrase–Chloride Interactions. The pH dependence of the ^{35}Cl nuclear magnetic resonance line widths of 0.5 M NaCl solutions of human B and C and horse B and C enzymes appears in Figures 1–4. The pH was varied by adding microliter amounts of concentrated NaOH or HCl and was measured after the recording of eight to ten spectra. The studies depicted in Figures 1 and 2 were performed on two separate preparations of the human B and C enzymes. Circles denote one preparation of the C enzyme while squares denote a separate preparation for the B enzyme. The various symbols in Figures 3 and 4 refer to separate titrations of the same stock solutions.

The pH of 0.5 M NaCl solutions of the human C enzyme was varied between ~5.3 and 9. In 0.5 M NaCl, at the probe temperature the protein precipitated at pH ~5.3. The pH of 0.5 M NaCl solutions of the human B enzyme was varied between 6 and 12; below pH 6 the protein precipitated. Solutions of horse carbonic anhydrase B, in 0.5 M NaCl, were clear over the range 4–11.3. The horse C enzyme was studied only over the pH range 6–9 owing to insufficient quantities of the protein. In all cases, including the bovine enzyme (Ward, 1969), the line broadening passes through a maximum and then decreases nearly to zero as the pH in increased. The various isozymes, however, do exhibit large differences in the apparent p*K*'s obtained from the titration curves. In this report, p*K* will be defined as that pH where the line broadening has decreased to one-half of its maximum value. The observed p*K*'s allow a classification of the isozymes from various sources into two groups. This grouping is identical with that obtained from a consideration of the specific activities of the various enzymes. The low specific activity forms both exhibit a p*K* which is approximately two p*K* units higher than the p*K* obtained for the high specific activity forms of carbonic anhydrase.

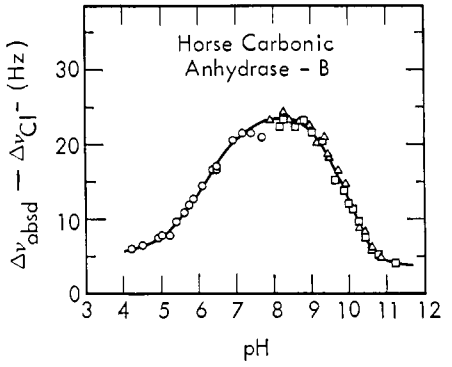

FIGURE 3: $\Delta\nu_{obsd} - \Delta\nu_{Cl^-}$ (Hz) *vs.* pH for a solution containing 1.28 mg/ml of horse carbonic anhydrase B in 0.5 M NaCl. The pK value is 9.85.

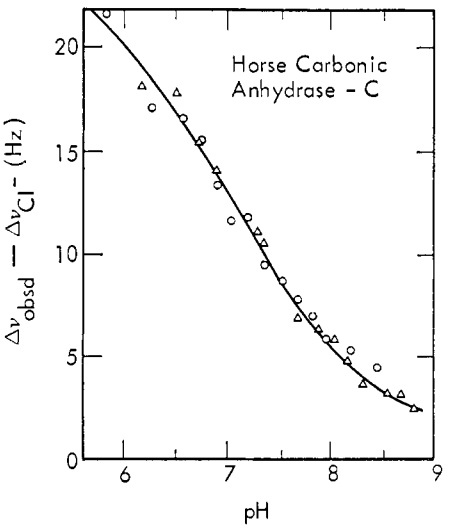

FIGURE 4: $\Delta\nu_{obsd} - \Delta\nu_{Cl^-}$ (Hz) *vs.* pH for a solution containing 1.27 mg/ml of horse carbonic anhydrase C in 0.5 M NaCl. The pK value is 7.23.

A previous study with bovine carbonic anhydrase (Ward, 1969) demonstrated that the observed pK depends on the chloride ion concentration. A similar dependence has also been observed for the human B and C enzymes and consequently the pH dependence of the ^{35}Cl broadening was studied for both isozymes over the range of 0.15–1.5 M NaCl. The variation of pK with chloride ion concentration follows a hyperbolic saturation curve for both the C and B enzymes with the maximum value reached at approximately 1.0 M chloride for the C enzyme. The pK for the B enzyme continued to increase with increasing chloride until the protein precipitated.

R. G. Khalifah (personal communication from J. T. Edsall) has pointed out that the pK dependence on chloride concentration can be analyzed in terms of competitive equilibria, by a slight modification of the analysis previously presented by Kernohan (1965). Denote the competitive binding of chloride by the enzyme by eq 2

$$E\text{-}Zn\text{-}OH_2 + Cl^- \rightleftharpoons E\text{-}Zn\text{-}Cl^- + H_2O \qquad (2)$$

with $K_i = [Cl^-][E\text{-}Zn\text{-}OH_2]/[E\text{-}Zn\text{-}Cl^-]$ and the acid–base forms of the enzyme by eq 3

$$E\text{-}Zn\text{-}OH_2 \rightleftharpoons E\text{-}Zn\text{-}OH^- + H^+ \qquad (3)$$

with $K_h = [H^+][E\text{-}Zn\text{-}OH^-]/[E\text{-}Zn\text{-}OH_2]$.

Let the fraction of chloride bound zinc sites be represented by α, *i.e.*, $\alpha = [E\text{-}Zn\text{-}Cl]/T_{E\text{-}Zn}$, where the total number of zinc sites, $T_{E\text{-}Zn}$, is given by

$$T_{E\text{-}Zn} = [E\text{-}Zn\text{-}OH_2] + [E\text{-}Zn\text{-}OH^-] + [E\text{-}Zn\text{-}Cl^-] \qquad (4)$$

then

$$\alpha = \frac{[Cl^-]/K_i}{1 + \frac{K_h}{[H^+]} + \frac{[Cl^-]}{K_i}} \qquad (5)$$

If $[H^+] \gg K_h$, then $\alpha = \alpha_{max}$. pK' is defined as that hydrogen ion concentration $[H^+]^*$ such that $\alpha = \alpha_{max}/2$. This gives $[H^+]^* = K_h/(1 + [Cl^-]/K_i)$ or

$$pH^* = pK' = pK_h + \log\left[1 + \frac{[Cl^-]}{K_i}\right] \qquad (6)$$

Thus a plot of observed pK' *vs.* log $[1 + ([Cl^-]/K_i)]$ should be a straight line with intercept pK_h. This treatment includes the related equilibria

$$E\text{-}Zn\text{-}OH^- + Cl \rightleftharpoons E\text{-}Zn\text{-}Cl^- + OH^-$$

for which Kernohan (1965) gives, for the bovine enzyme, a value of K_i of 1000 mM compared with 30 mM for the K_i of eq 2. This treatment has been applied to the chloride data of bovine carbonic anhydrase (Ward, 1969) by Khalifah. He obtained a straight-line plot which yielded a value for pK_h of 6.2. Kernohan (1965) studied the effect of anions on the enzymic activity of bovine carbonic anhydrase and concluded that the enzymic process involves only one group which has a pK value of 6.35 in close agreement with the nuclear magnetic resonance result. The value obtained from extrapolation of the hyperbolic saturation curve of apparent pK *vs.* chloride concentration to zero chloride was 7.0.

This treatment has been applied to the human enzymes using K_i values of 51 and 67 mM from the work of Verpoorte *et al.* (1967) for the B and C enzymes, respectively. The data plotted in Figure 5 were obtained from a number of titrations of three different enzyme preparations. Each pK value was obtained from line width *vs.* pH measurements at a fixed chloride concentration as previously described. The chloride concentration was varied between 0.15 and 1.5 M. The lower limit was determined by signal-to-noise considerations in the line-width measurements, while the higher limit was set by protein solubility. In order to obtain reasonable chloride line widths the protein concentration was changed proportionally as the chloride ion concentration was varied. The straight lines plotted in Figure 5 have been fitted to the experimental points by a least-squares analysis. The pK_h's obtained from the intercepts are 6.4 and 8.19 for the human C and B carbonic anhydrases, respectively.

This analysis neglects ionic strength effects. Changes in ionic strength could affect the structure of the protein as well as the values of the constants used in expression 6. In an attempt to evaluate possible ionic strength effects on the

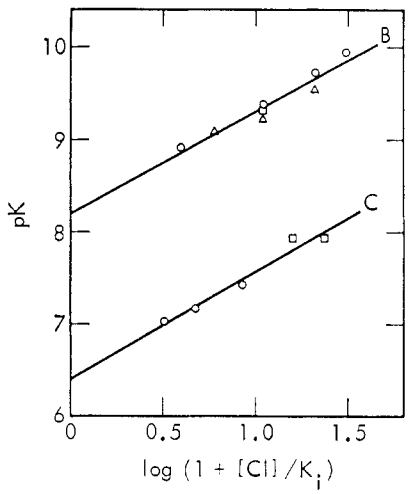

FIGURE 5: A plot of measured pK *vs.* log (1 + [Cl]/K_i) for human carbonic anhydrase B and C. The K_i values are 51 and 67 mM for the B and C enzymes, respectively. The intercepts represent the value of pK_h for zero chloride and are 8.19 and 6.4 for the B and C proteins.

observed pK's, a series of measurements were made on both the human B and C enzymes in which the ionic strength was held constant while the chloride concentration was varied. The ionic strength was maintained at a value of 1.0 by the addition of Na_2SO_4. This salt was chosen because of the known inhibition of carbonic anhydrases by essentially all monovalent anions. The chloride concentration was varied between 0.15 and 1.0 M. For both the B and C enzymes the pK's were not, within the experimental error, ±0.1 unit, affected by the presence of Na_2SO_4. The molar relaxivity of human B and C carbonic anhydrases was also independent of ionic strength. As before, Ward (1969), molar relaxivity is defined as the increase in chloride line width in Hz divided by the molar concentration of the enzyme.

It is apparent from Figures 1–3 that the chloride broadening reaches a maximum value and then decreases as the pH is increased or lowered. We have proposed that the decrease in chloride broadening as the pH is raised, is due to hydroxide competition for the zinc site. The decrease is broadening

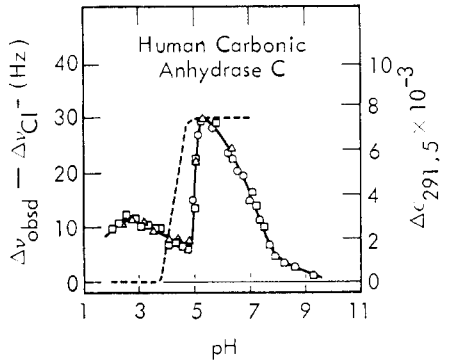

FIGURE 6: $\Delta\nu_{obsd} - \Delta\nu_{Cl^-}$ (Hz) *vs.* pH for a solution containing 0.3 mg/ml of human carbonic anhydrase C in 0.15 M NaCl and $\Delta\epsilon$ (dashed curve) as a function of pH at 291.5 mμ in 0.15 M NaCl (redrawn from Riddiford, 1965).

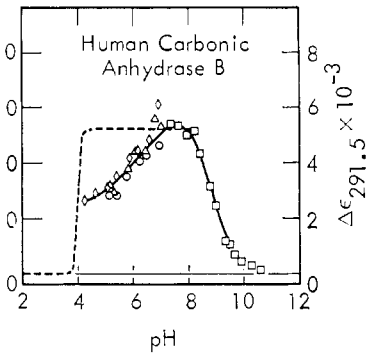

FIGURE 7: $\Delta\nu_{obsd} - \Delta\nu_{Cl^-}$ (Hz) *vs.* pH for a solution containing 0.69 mg/ml of human carbonic anhydrase B in 0.15 M NaCl and $\Delta\epsilon$ (dashed curve) as a function of pH at 291.5 mμ in 0.15 M NaCl (redrawn from Riddiford, 1965). The diamonds are for a solution containing 0.53 mg/ml (see text).

as the pH is lowered is, however, another matter. Only in the case of horse B carbonic anhydrase was it possible to lower the pH to 4 in 0.5 M NaCl without protein precipitation. It was possible to lower the pH further than depicted in Figures 1 and 2 for the two human enzymes if 0.15 M NaCl and lower protein concentrations were used (Figures 6 and 7). Riddiford (1965) has reported acid ultraviolet difference spectra in 0.15 M NaCl at room temperature and her data (dashed lines) are presented for comparison.

The chloride line-width measurements in the acid region are notably different for the two enzymes. The chloride measurements for the C enzyme closely parallel those of Riddiford (Figure 6). The small displacement in the curves may be due to differences in temperature, 32.9° *vs.* room temperature. The sharp change in the chloride line width at pH 5 for the C enzyme is irreversible. If the pH of the 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 about 6, where the protein precipitates. The chloride line widths as a function of pH for the B enzyme, in the acid region, do not parallel Riddiford's curve (Figure 7). The decrease in chloride 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 human carbonic anhydrase B undergoes an irreversible conformational change (Riddiford, 1965). The diamonds in Figure 7 are points measured for a solution containing ~0.53 mg/ml of protein. This concentration allowed a lower pH to be reached before protein precipitation took place. The measured line widths were normalized accordingly.

Available Zinc Coordination Sites. Cyanide ion titration studies have been used previously to demonstrate that there is one available zinc coordination site for chloride ion in the bovine enzyme. The results of a cyanide ion titration of horse carbonic anhydrase C is shown in Figure 8. This titration is somewhat typical of all the isozymes studied and agrees with the earlier bovine enzyme results (Ward, 1969). The extrapolated end point is at a molar ratio of one cyanide ion to one carbonic anhydrase molecule. One to one end points were also obtained for cyanide titrations of horse B and the two human enzymes.

Sulfonamide Inhibition. Sulfonamides are highly specific and potent inhibitors of carbonic anhydrase. Previous studies

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

[a] The molar relaxivity (see text) is the maximum observed value in 0.5 M NaCl.

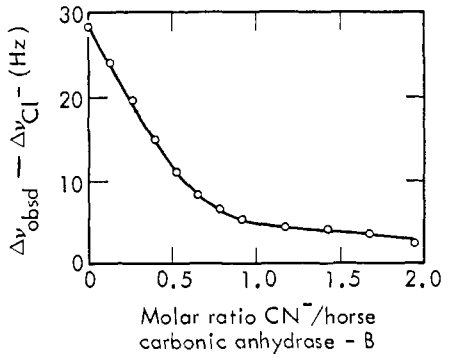

FIGURE 8: $\Delta\nu_{obsd} - \Delta\nu_{Cl^-}$ (Hz) *vs.* molar ratio of CN^-/horse carbonic anhydrase B for a solution of 0.5 M NaCl, 5.5×10^{-5} M NaCl, 5.5×10^{-6} M carbonic anhydrase, and 0.05 M glycine buffer (pH 8.8).

with the bovine enzyme have shown that acetazolamide binds to the enzyme in such a manner that chloride either does not interact with the zinc atom of the exchange of aqueous chloride with zinc-bound chloride is prevented. Acetazolamide titrations of the various carbonic anhydrases reveal that the chloride broadening is reduced to that of the cyanide-inhibited enzyme at a molar ratio of one acetazolamide to one carbonic anhydrase molecule. Acetazolamide titrations of the two human enzymes are shown in Figure 9. The titration was performed at pH 8.0 for the B enzyme and at pH 6.85 for the C enzyme. The difference in the sharpness of the end points reflects the fact that human C binds acetazolamide much more strongly than the B form (Verpoorte *et al.*, 1967). The binding of acetazolamide varies little in this pH range (Coleman, 1967).

Molar Relaxivity. The molar relaxivity of carbonic anhydrase as defined previously is a function of the pH and the chloride ion concentration. A maximum value can be estimated for each enzyme at a given chloride concentration. These values, along with the observed pK for the various enzymes in 0.5 M NaCl, are listed in Table I. The classification according to group I or II is by pK. The pK value more clearly distinguishes high from low specific activity forms of the enzyme than does the molar relaxivity parameter. It does appear, however, that the high specific activity forms of carbonic anhydrase are generally more effective in increasing the ^{35}Cl nuclear magnetic relaxation rate.

Apocarbonic Anhydrase. It has been reported earlier that bovine apocarbonic anhydrase has little effect on the ^{35}Cl line width and further that Zn(II) can be back-titrated into the enzyme to yield both an active protein and a chloride broadening which has a maximum value at a ratio of one Zn(II):apocarbonic anhydrase. Essentially identical results have been obtained with the human B apocarbonic anhydrase. These data will be published at a later time.

Discussion

Carbonic Anhydrase Isozymes. The first indication that carbonic anhydrase existed in multiple forms in the intact red blood cell came from the work of Lindskog (1960) in which he demonstrated that two forms of the enzyme could be separated from bovine erythrocytes. The following year Nyman (1961) reported the separation of three distinct forms of carbonic anhydrase from human erythrocytes. These distinct proteins are now known as the A, B, and C forms of the enzyme.

The major human carbonic anhydrases, B and C, differ strikingly from each other in specific activity, in amino acid composition, and in immunological specificity. The specific activity for CO_2 hydration is far larger for human C than for B, although the latter is present in erythrocytes in much larger amounts. Each form does, however, contain one tightly bound zinc atom per molecule, which can be reversibly removed by treatment with chelating agents. Enzyme activity is lost on zinc removal and is fully regained on addition of zinc.

Horse erythrocytes also yield two major forms of carbonic anhydrase (Furth, 1968) which are similar to the human enzymes in that the one present in the greatest abundance, B, is the less active. The high specific activity horse enzyme, C, in itself, is particularly unusual in that it possesses a very alkaline isoelectric point; the protein still retains a net positive charge at pH 10.

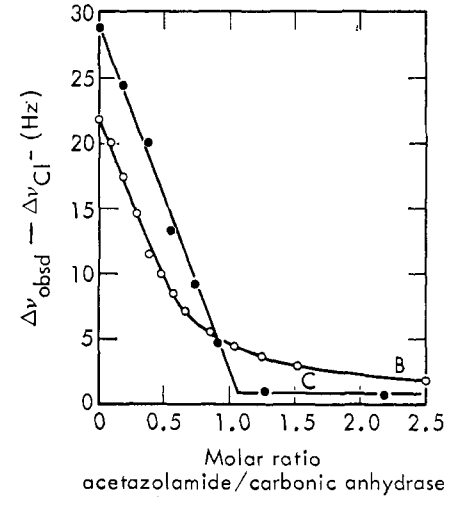

FIGURE 9: $\Delta\nu_{obsd} - \Delta\nu_{Cl^-}$ (Hz) *vs.* molar ratio of acetazolamide/carbonic anhydrase for a solution of 0.5 M NaCl, 0.05 M Tris, 7.0×10^{-5} M human B (open circles, pH 8.0), and 3.9×10^{-5} M human C (closed circles, pH 6.85).

Bovine carbonic anhydrase consists primarily of one protein which has been designated form B. A rather minor component can also be isolated, but its characteristics are quite similar to the major component. The designation of the letter B for the bovine protein is unfortunate in that greater similarities in both specific activity and amino acid sequence are found between the bovine enzyme and human carbonic anhydrase C than human B (Nyman *et al.*, 1968).

Zinc Studies. Although carbonic anhydrase catalyzes the hydration of a variety of substrates, the physiologically important reaction is the reversible hydration of carbon dioxide to form bicarbonate. The review of Edsall (1968) is recommended for an excellent discussion of this and the other reactions catalyzed by carbonic anhydrase. Although the kinetic constants of the various isozymes for a given reaction are quite different, the pH–rate profiles are similar in that they can be adequately described by a sigmoid curve which represents the ionization of a single group. The inflection point of these curves, as measured by the esterase reaction, does, however, vary almost 1 pH unit as a function of species and isozyme (Coleman, 1967). Because of the stringent zinc requirement of the enzyme, a mechanism has evolved in which zinc plays a major role in the ionization of this single group (Bradbury, 1969).

To better understand the mechanism of action of carbonic anhydrase and the individual isozymes themselves more information is needed concerning the nature of the zinc ion and its environment. Since previous ^{35}Cl magnetic resonance studies on the bovine enzyme have shown that the increased relaxation rate of ^{35}Cl nuclei in the presence of the protein is due primarily to a zinc–chloride interaction amplified by the rotational correlation time of the protein molecule (Ward, 1969), studies were initiated on the separated human and horse isozymes.

A comparison of the pH–chloride line-width profiles in 0.5 M NaCl for the two human enzymes, the two horse enzymes and that of the bovine enzyme reported earlier (Ward, 1969) reveals that the isozymes can be placed in one of two groups which correspond to their respective specific activities (Figures 1–4 and Table I). The high specific activity forms cluster in a range of pK's of ~7.2–7.4 in 0.5 M NaCl, while the low specific activity forms have pK's in the range 9.2–9.8. These results imply that the environments of the zinc atoms of the various carbonic anhydrase isozymes differ in a manner sufficient to produce these different pK's.

Verpoorte *et al.* (1967), Lindskog (1966), and Kernohan (1964) have shown for both the human and bovine enzymes that the apparent pK of the group on the enzyme governing its catalytic functions shifts upward in the presence of monovalent anions. We have therefore measured the apparent pK as a function of total chloride concentration for the two human enzymes and analyzed the results in terms of the equilibria outlined by Khalifah. The least-square analysis of the data, which appears in Figure 5, extrapolates to a pK_h of 8.19 for the low specific activity or B form and to 6.4 for the high specific activity or C form of the protein.

Although the extrapolation is rather long, the observed pK's are the results of a number of titrations of three different enzyme preparations, and each pK is considered to be accurate to ±0.1 unit or less. Although this treatment ignores ionic strength effects, the results mentioned earlier on the lack of an effect of an added noninhibitory electrolyte on the pK's indicates that ionic strength effects are small over the range studied.

Comparison of Chloride and Kinetic Observations. The pK_h value of 6.4 of the human C enzyme is in close agreement with that of 6.2 mentioned earlier for the bovine enzyme. These results suggest that the zinc atom of all high specific activity forms of carbonic anhydrase should exhibit pK_h's in the range of ~6.2–6.4. Similarly, the low specific activity forms of carbonic anhydrase should exhibit pK_h's near the value observed for the human B enzyme, 8.19.

The pK_h values, 6.2–6.4, are in good agreement with the kinetically obtained values for the high specific activity carbonic anhydrases (Kernohan, 1965). For example, Kernohan's value of 6.3 for the bovine enzyme is for zero chloride, whereas he also reports a value of 6.9 for solutions 80 mM in chloride. We estimate a value of 6.75 for an 80 mM chloride solution from a plot of pK *vs.* log $(1 + [Cl]/K_i)$ for the bovine data (Ward, 1969).

The agreement between the chloride data and kinetic data for human B carbonic anhydrase, the low specific activity form, is less readily apparent. Bradbury (1969) notes that five different investigators have studied the pH–rate profile for catalysis and the binding of anions for the human B enzyme. Verpoorte *et al.* (1967) report that the pH–activity curve for ester hydrolysis is sigmoid with an inflection point at pH 7.3. Whitney *et al.* (1967) confirmed Verpoorte's data on the pH–rate profile, observing a pK value of ~7.4. The data of Lindskog and Nyman (1964) and Magid (1968) also agrees well with this value.

Bradbury (1969) further points out that only the data of Coleman (1967) do not concur. Coleman studied the competition of acetate, azide, cyanide, sulfide, and cyanide with tritium-labeled acetazolamide for the binding site at the active center. He reported that the binding of 1 equiv of sulfide or cyanide to human carbonic anhydrase B at pH values below the pK values of H_2S and HCN is accompanied by the release of H^+ ions. Over the pH range in which the inhibitors are in the form CN^- and HS^-, binding is accompanied by the appearance of OH^- ions. His experimental data fit theoretical curves constructed by assuming a single additional H^+ dissociation associated with the protein, coupled with the metal ion, and described by a pK of 8.1. Coleman further observed that the esterase pH–rate profile for human carbonic anhydrase B exhibited an inflection point with a pK of 8.1. Bradbury, however, points out that Coleman's pH–rate profiles were determined in the presence of 20 mM acetate, which inhibits the enzyme with a K_i value of 22 mM. Since monovalent anions cause an upward shift in the apparent pK of the group on the enzyme governing the catalytic function, there appears to be some uncertainty as to whether kinetic studies and chloride binding and/or complexometric titrations yield the same pK for human carbonic anhydrase B. This variance may relate to the importance of an amino acid residue near the active site. Bradbury (1969) and others have demonstrated the importance of a reactive histidine near the active site of human carbonic anhydrase B. No such reactive histidine has been found for the human C enzyme.

Stoichiometry of Chloride Bindings. The stoichiometry of the zinc–chloride interaction in the various carbonic anhydrases is also important from a mechanistic and comparative viewpoint. Cyanide titration of the bovine enzyme

has shown that there is one zinc coordination site available for cyanide binding and thus presumably for chloride ion, water, and substrate interactions (Ward, 1969). A representative cyanide titration of one of the enzymes, horse carbonic anhydrase B, is given in Figure 8. Again the extrapolated cyanide end point is at a molar ratio of one cyanide ion per carbonic anhydrase. This result pertains to all the carbonic anhydrases studied in this investigation. These results are in agreement with the crystallographic studies of Fridborg *et al.* (1967) who have shown for the human C protein that the zinc atom resides in the base of a cavity of the enzyme with one coordination site projecting outward into the cavity. The remaining three coordination sites of the zinc are occupied by amino acids of the protein.

Protein Conformation and Zinc–Chloride Interactions. Although 0.5 M NaCl is a convenient concentration for a comparison study of the human and horse enzymes by ^{35}Cl nuclear magnetic resonance, it has been necessary to reduce the chloride concentration to 0.15 M in order to study the acid dependence of the chloride broadening for the two human enzymes. Fortunately, acid ultraviolet difference spectra data have been reported for the two human enzymes in 0.15 M NaCl (Riddiford, 1965). It is therefore possible to examine the relation between the overall structure of the protein and the interaction of chloride ions with the zinc ion.

As can be seen from Figure 5, the chloride line width for the C enzyme closely parallels the changes in the ultraviolet difference spectrum. The ultraviolet difference spectrum indicates that a change in the overall conformation of the protein takes place at ~pH 4.5. It has been suggested that perhaps the main contribution to the chloride broadening is the correlation time for molecular motion of the protein (Ward, 1969). In order for this correlation time to shorten, while the protein appears to be unfolding, the coordination of the zinc to the protein must change. The zinc–protein coordination therefore become less rigid in nature. The exact nature of this change is unknown, however, but the irreversibility of this change, as measured by chloride line broadening, has been noted.

The B enzyme exhibits an entirely different behavior in that the chloride broadening decreases gradually and reversibly until pH 4.3 is reached. At this pH the protein precipitated under the conditions of the experiment. Riddiford (1965) notes that human carbonic anhydrase B loses enzymatic activity and undergoes an irreversible conformational change near pH 4. The reversible change in chloride broadening between pH 7.5 and 4.3 could be explained by competition of a proton for one of the zinc coordination sites yielding a less rigid zinc environment. In the pH region, 5–7, the zinc remains firmly bound to the protein (J. T. Edsall, personal communication) and therefore the decrease in chloride line width cannot be attributed to removal of zinc.

Enzymatic Mechanism. The simplest mechanism to consider is that in which a water molecule occupies a position within the coordination sphere of the zinc. This form of the enzyme would be inactive in the hydration of carbon dioxide. As the pH is increased, the water molecule ionizes to form a protein–zinc–hydroxide ion complex which is the active form of the enzyme. The ionization of the zinc–enzyme–water molecule complex would thus be described by eq 3. Bradbury (1969) has discussed the available kinetic data at length and concluded that a pK value of 7.1–7.4 should be assigned to this equilibrium. This range of values is low compared with the pK of 9 assigned to the dissociation of a proton from water liganded to zinc ions in an aqueous environment (Chaberek *et al.*, 1952). Bradbury (1969), however, suggests that the environment of the zinc in the metalloenzyme greatly increases the tendency of the water molecule to lose a proton. Certainly the strong binding of anions to the enzyme-bound zinc compared with aqueous zinc ions constitutes evidence for this view. Pocker *et al.* (Pocker and Meany, 1965; Pocker and Stone, 1967, 1968a,b; Pocker and Dickerson, 1968) have, however, proposed an alternative interpretation, they assign the pK near neutrality to an imidazole nitrogen. In their mechanism an unprotonated histidine base promotes the transfer of a water molecule from the coordination sphere of the zinc to the carbonyl carbon of carbon dioxide with the subsequent formation of bicarbonate.

If one accepts the general belief that the role of the metal cation in the protein is to increase the acidity of a bound water molecule (Jencks, 1969), than a possible explanation for the difference in pK's for the high and low specific activity forms of carbonic anhydrase can be suggested. It is proposed that the reactive histidine, present near the active or zinc site in human carbonic anhydrase B, forms a hydrogen bond with the water molecule liganded to the zinc. This hydrogen bond hinders rather than helps the formation of the enzyme–zinc–hydroxide complex. The lack of such a histidine near the active site of the human C enzyme allows the formation of the enzyme–zinc–hydroxide complex at a lower pH. This interpretation, however, does not clear up the aforementioned discrepancy between the kinetic and complexometric studies of human carbonic anhydrase B.

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Studies on the Substrates of D-Fructose 1,6-Diphosphate Aldolase in Solution*

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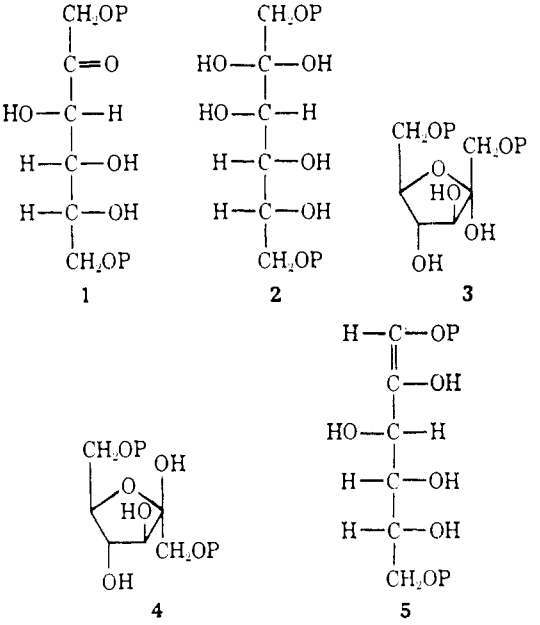
ABSTRACT: Ketose mono- and diphosphates have been examined by infrared, ultraviolet, and nuclear magnetic resonance spectroscopy in deuterium oxide solutions. Those which have a hydroxyl group at C-5 (D-fructose 1,6-diphosphate) or C-6 (D-*glycero*-D-*altro*-octulose 1,8-diphosphate) are in the furanose and pyranose ring forms, respectively. Ketose phosphates which cannot exist in ring forms are in the free keto and hydrated keto forms in solution. The proportion of the keto form at 25° for the phosphates examined is given in parentheses in the following list: 5,6-dideoxy-D-*threo*-hexulose 1-phosphate (96%), D-*erythro*-pentulose 1,5-diphosphate (84%), 1,5-dihydroxy-2-pentanone 1,5-diphosphate (84%), 1,3-dihydroxy-2-propanone phosphate (55%), D-fructose 1,6-diphosphate (<1.7%), D-*glycero*-D-*altro*-octulose 1,8-diphosphate (none). The K_m values of the ketose diphosphates for rabbit muscle aldolase relate to the distance between the phosphate groups in the predominant form in solution.

D-Fructose 1,6-diphosphate ($FruP_2$), the substrate of aldolase, would be expected to exist in aqueous solution as a mixture of forms **1, 2, 3, 4,** or **5.** It has usually been assumed (Rutter, 1961) that it exists predominately in a furanose form (3 or 4), since in the monosaccharides, ring forms are usually more stable than acyclic forms. However, no direct evidence has been obtained to demonstrate that this is the case. From an examination of ultraviolet spectra, McGilvery (1965) concluded that $FruP_2$ dianion exists predominately as the free keto form **1,** and the tetraanion as the enediol form **5.** The established importance of acyclic forms of $FruP_2$ and other phosphorylated ketoses as intermediates in enzymatic reactions makes their relative abundance in solution an important consideration in describing the mechanism of enzyme action (Mehler and Cusic, 1967; Koshland and Neet, 1968; Knowles and Pon, 1968).

The acyclic ketose phosphates, 1,3-dihydroxy-2-propanone phosphate (**6**), 5,6-dideoxy-D-*threo*-hexulose 1-phosphate (**7**), D-*erythro*-pentulose 1,5-diphosphate (**8**), and 1,5-dihydroxy-2-pentanone 1,5-diphosphate (**9**), and the potentially cyclic $FruP_2$, and D-*glycero*-D-*altro*-octulose 1,8-diphosphate (**10**) have been examined.



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