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Proton Magnetic Resonance Studies of Carbonic Anhydrase. II. Group Controlling Catalytic Activity†

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ABSTRACT: The seven resonances observed in the histidine region of the proton magnetic resonance (pmr) spectrum of human carbonic anhydrase B and reported in the preceding paper are studied in the presence of sulfonamide, azide, cyanide, and chloride inhibitors and in metal-free, cadmium substituted, cobalt substituted, and carboxymethylated forms of the enzyme. Results indicate that the two resonances that move downfield with increasing pH and the two that do not move with pH reflect residues located at the active site. The first two resonances are assigned to the same titratable histidine whose pK value of 8.24 corresponds to that of the group controlling catalytic activity. Addition of anions or sulfonamides, removal of zinc, or substitution of cadmium for zinc at the active site, procedures known to abolish enzymatic activity, prevent titration of this residue. Partial inhibition of carbonic anhydrase by chloride selectively increases the pK value of the group controlling catalytic activity and of the histidine with pK = 8.24. Experiments with metal-free and cadmium carbonic anhydrases and comparisons with model systems suggest that this histidine is bound to the metal ion at high pH; at low pH this complex appears to dissociate as protons compete with the metal for the imidazole group. It is proposed that ionization of the group controlling catalytic activity represents loss of the pyrrole proton of this neutral ligand when it binds to Zn(II), forming an imidazolate anion and juxtaposing a strong base and a powerful Lewis acid at the active site. When bound to zinc as an anion, this histidine can act as a general base catalyst in the hydration of carbon dioxide and be replaced as a metal ligand by an oxygen of the substrate in the course of the reaction. The histidine–metal complex is thought to exist in a strained configuration in the active enzyme so that its imidazole–metal bond is readily broken on addition of substrates or inhibitors. This model is consistent with the available data on the enzyme and is discussed in relation to alternative proposals.

Carbonic anhydrase accelerates the reversible hydration of carbon dioxide by a factor of 10^9 (Prince and Woolley, 1972), giving it one of the highest turnover numbers known for an enzymatic reaction (Edsall, 1968)

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

Although hydration of carbon dioxide has been extensively investigated using model systems (Garg and Maren, 1972; Dennard and Williams, 1966), and detailed studies of carbonic anhydrase have included its high resolution X-ray structure (Kannan *et al.*, 1971; Liljas *et al.*, 1972) and primary sequence (Andersson *et al.*, 1972; Lin and Deutsch, 1973; Henderson *et al.*, 1973; Lin and Deutsch, 1974; Laurent-Tabusse *et al.*, 1972), neither the mechanism for the enzymatic reaction nor the identity of the group(s) controlling activity has been definitively established.

The pH dependencies of enzymatic activity and of the visible absorption spectrum of catalytically active cobalt substituted carbonic anhydrase are described by titration curves with pK values near neutrality and are thought to reflect ionization of a group on or near Zn(II) controlling catalytic activity (Kernohan, 1964; Lindskog, 1963; Lindskog and Nyman, 1964; Taylor *et al.*, 1970; Khalifah, 1971). Both titrations are abolished by addition of anion or sulfonamide inhibitors. Potentiometric studies of the addition of Zn(II) to the metal-free enzyme suggest that this group forms an additional ionized ligand to the metal ion at high pH (Lindskog, 1963). Since several histidines are present at the active site of HCA-B[1] (Kannan *et al.*, 1971; Liljas *et*

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[1] Abbreviations used are: HCA-B and HCA-C, human carbonic anhydrase B and human carbonic anhydrase C, respectively.

Table I: Ionization Constants of Histidine Residues of HCA-B.

	Resonances							
	1	2	3	4	5	8	9	10
Native enzyme	8.2	7.23	6.98	6	8.24	NO[a]	NO	NO
Enzyme inhibited by azide	NT[b]	7.28	6.98	5.93	NT	6.18	NO	NO
Enzyme inhibited by cyanide	NT	7.24	6.98	6	NT	NO	NO	NO
Enzyme partially inhibited by chloride	9.0	7.26	7.00	6.09	8.9	NO	NO	NO
Enzyme partially inhibited by Fremy's salt	I[c]	7.24	6.99	6	I	NO	NO	NO
Enzyme inhibited by acetazolamide	NT	7.26	7.00	6	NO	7.13	NO	NO
Carboxymethylated enzyme	NT	7.28	7.00	6	NO	NO	NO	NO
Apoenzyme	NO	7.24	7.00	6	NT	NO	5.70	4.9
Cadmium enzyme	NT	7.23	6.99	6	NT	NO	NO	5

[a] NO = resonance not observed. [b] NT = nontitrating. [c] I = indeterminate.

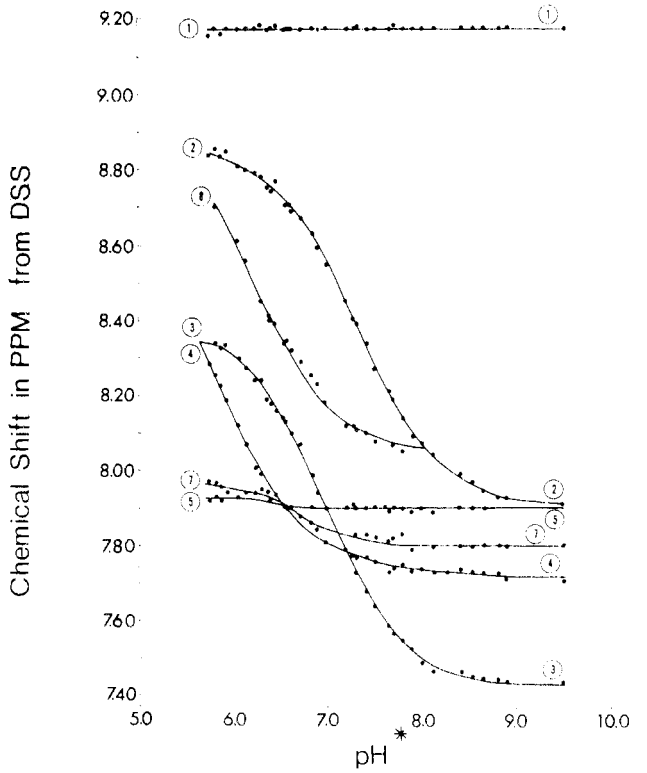

FIGURE 1: pH dependence of resonances in the histidine region of the pmr spectrum of HCA-B inhibited by 50 mM azide. Numbering scheme corresponds to that used in Figure 2 of the preceding paper. Samples were 2 mM in protein and run using sodium azide as the supporting electrolyte. Curves for resonances 2, 3, 4, and 8′ were calculated using the Henderson–Hasselbalch equation and represent the best fit to the experimental points. Curves describing resonances 5 and 7 were hand drawn. The curves for peaks 5 and 7 at low pH are only partly resolved.

al., 1972), and since several of the histidines reported in the preceding paper possess pK values that warrant their consideration for assignment to the group controlling enzymatic activity, the goal of the present study is to further characterize the latter residues, to identify those located at the active site, and to determine their role, if any, in the catalytic mechanism.

Experimental Section

Chemicals. Spectropure cobaltous sulfate was purchased from the Johnson, Matthey Company (London) while cadmium sulfate was a Fisher Certified reagent. All other materials and procedures are as described in the preceding paper.

Modified Enzymes. Metal-free carbonic anhydrase was prepared by a variation of the method of Lindskog and Malmstrom (1962). A concentrated solution of enzyme was dialyzed against 4 l. of 0.1 M sodium acetate (pH 5.2) containing 0.01 M *o*-phenanthroline for 6 weeks with five changes of the external solution, all at 4°. Atomic absorption analysis indicated less than 5% of the total zinc content remaining. Full activity was restored on addition of 1 equiv of zinc sulfate.

Cobalt and cadmium carbonic anhydrases were prepared by direct addition of 0.95 equiv of the respective sulfates to solutions containing known concentrations of the apoenzyme in deuterium oxide near pH* 7, (hereafter referred to simply as pH). Samples were allowed to stand for at least 2 days prior to use.

Histidine-200 at the active site of carbonic anhydrase was carboxymethylated using iodoacetate (Bradbury, 1969). This modified enzyme, a gift from Dr. M. E. Fabry, showed markedly reduced catalytic activity using *p*-nitrophenyl acetate as substrate.

Results

Effect of Inhibitors. AZIDE. The pH dependence of resonances in the histidine region of the spectrum of HCA-B in the presence of 50 mM azide, a concentration sufficient to completely inhibit the enzyme, is shown in Figure 1 and Table I. The spectrum of the free enzyme changes progressively as increasing amounts of azide are added until the ratio of inhibitor to enzyme reaches a value of approximately 1.6. Interaction of azide with HCA-B is rapid on the proton magnetic resonance (pmr) time scale, allowing resonances to be correlated on the free and azide-inhibited states. Resonances 2, 3, and 4 of the free enzyme (Figure 2 of preceding paper) are essentially unaffected and have been joined by a fourth, number 8′. Although the new resonance is not observed above pH 8, the available data can be fitted by a theoretical titration curve and give a pK value of 6.18. The chemical shift, pH dependence, and T_1 value of this peak are comparable to those of the other three. Peak 7 is now observed at low and high pH, and resonance 6 has disappeared. Addition of Fremy's salt at low pH selectively broadens peaks 5, 7, and 8.

Resonances 1 and 5 of the free enzyme lose their pH de-

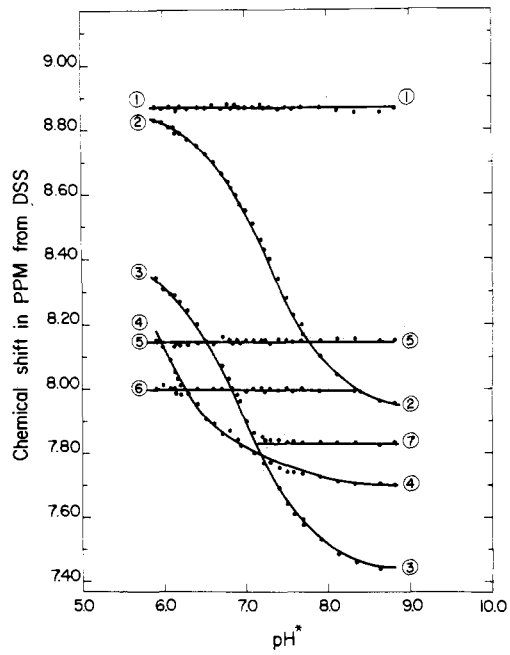

FIGURE 2: pH dependence of resonances in the histidine region of the pmr spectrum of HCA-B inhibited by 10 mM cyanide.

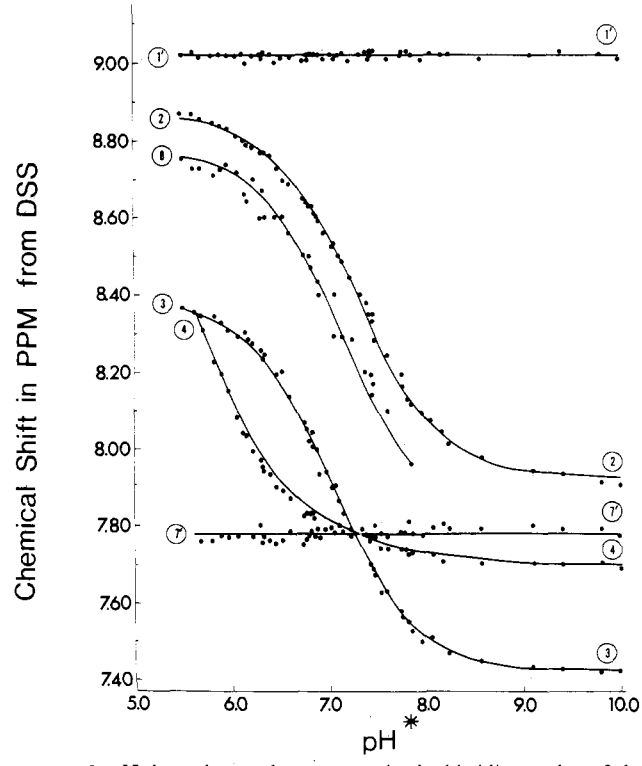

FIGURE 3: pH dependence of resonances in the histidine region of the pmr spectrum of HCA-B inhibited by acetazolamide.

pendence in the inhibited enzyme; the former moving downfield by 0.33 ppm from its position at low pH and the latter upfield by approximately 0.15 ppm (Figure 1). This change in chemical shift is proportional to the concentration of bound azide within the limits given above, and addition of acetazolamide produces a spectrum identical with that for the enzyme found in the presence of the sulfonamide alone. At pH 6.2, 0.86 mM of free azide is required to produce a half-maximal change in the chemical shift of both resonances and agrees well with the azide inhibition constant of 0.6 mM for the bovine enzyme at pH 5.5 (Riepe and Wang, 1968). The inhibition constant for binding of azide to carbonic anhydrase increases with increasing pH (Lindskog, 1966), and the total amount of azide required to produce a half-maximal change in the chemical shift of resonances 1 and 5 increases with pH in a comparable fashion.

CYANIDE. The spectrum of HCA-B with cyanide (Figure 2, Table I) closely resembles that of the uninhibited state (Figure 2 of preceding paper). Interaction of cyanide with HCA-B also appears to be short on the pmr time scale but is difficult to investigate because of the relatively small changes in chemical shift involved. Resonances 2, 3, 4, and 7 of the free enzyme are unaffected while 6 moves slightly downfield. Peaks 1 and 5 again lose their pH dependence, and the chemical shifts of these resonances move downfield by 0.03 and 0.05 ppm, respectively, from their values at low pH in the free enzyme. The spectra differ considerably from those produced by azide, notably in the presence and absence of peaks 6 and 8, respectively. Although 2:1 cyanide–enzyme complexes have been reported in electron paramagnetic resonance studies of the cobalt enzyme (Grell and Bray, 1971; Taylor and Coleman, 1971), no change in this region of the pmr spectrum of the zinc enzyme was observed on increasing the ratio of cyanide to enzyme.

ACETAZOLAMIDE. The changes produced in the spectra of HCA-B in the presence of a variety of sulfonamides constitutes the subject matter of the third paper of this series and will be discussed in detail therein. Results with acetazolamide (Figure 3 and Table I) are in many ways typical. Resonances 2, 3, and 4 of the free enzyme are unaffected while the behavior of peaks 1, 5, 6, and 7 has been altered. Acetazolamide exchanges slowly with the protein on the pmr time scale as demonstrated by the observation of two small peaks to low field in a half-inhibited solution of the enzyme. These peaks have chemical shifts equal to those observed for resonance 1 in the presence and absence of inhibitor. Attempts to correlate resonances in the presence and absence of acetazolamide must therefore be made by comparisons with the anion data where the inhibitor exchanges rapidly with the enzyme. When this is done, peak 1 (1′)[2] again seems to move downfield and lose its pH dependence while 5 and 6 disappear, being replaced by a cluster of resonances to high field (not shown) whose behavior is difficult to follow or interpret. Resonance 7 (7′) appears to shift slightly downfield and to be detectable over the entire pH range. As in the presence of azide, a new pH dependent peak (number 8) appears below pH 8, reflecting a group having pK value of 7.13. Its area, chemical shift, pH dependence, and T_1 value are comparable to those of peaks 2, 3, and 4. Comparison with published results (Cohen *et al.*, 1972) indicates that the pK of the group reflected by this peak shifts to lower values as ionic strength is increased, suggesting proximity of the parent residue to an anionic group(s) on the protein.

CHLORIDE. The pH dependence of the pmr spectrum of HCA-B in the presence of 100 mM chloride, a concentration of anion sufficient to produce partial inhibition of the enzyme (K_I = 0.051 M at pH 7.3 (Verpoorte *et al.*, 1967), is shown in Figure 4 and Table I. Spectra so produced closely resemble those of the uninhibited enzyme (Figure 2 of preceding paper). The data for peak 4 but not 1 and 5 fit a

[2] Suspected identity of peaks in states of the enzyme not interconverting rapidly on the pmr time scale is indicated using primed numbers (1′, 2′, etc.).

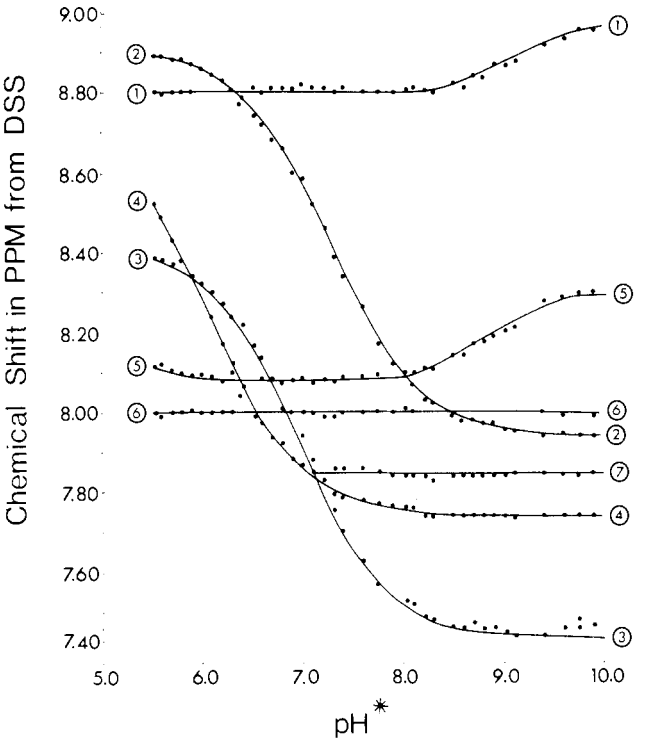

FIGURE 4: Titration of the histidine resonances of HCA-B in the presence of 100 mM chloride. Enzyme concentration was 2.8 mM.

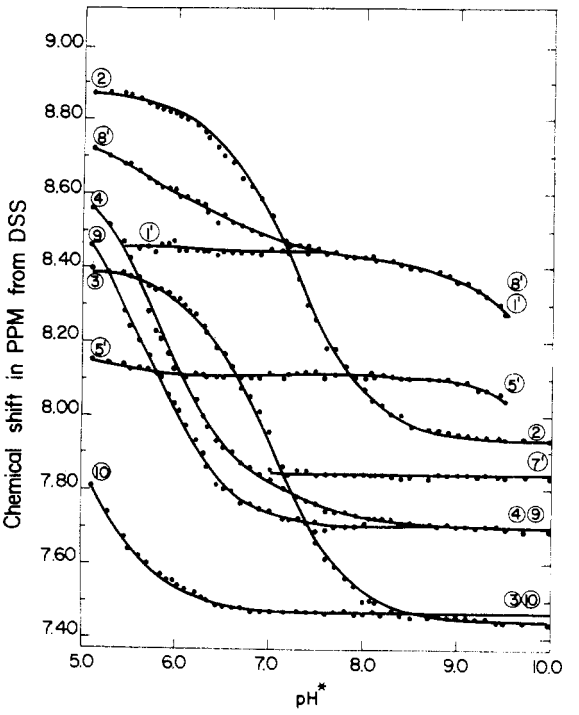

FIGURE 5: pH dependence of the pmr spectrum of the low field aromatic region of metal-free HCA-B.

theoretical titration curve, and the pK values of the groups reflected by these resonances change to 6.09, 9.0, and 8.9, respectively. pK values of the parent residues of peaks 1 and 5 are determined from the points of half-maximal change in chemical shift.[3]

Modifications of Carbonic Anhydrase. CARBOXYMETHYLATION OF HISTIDINE. When histidine-200 at the active site of HCA-B is carboxymethylated using iodoacetate (Bradbury, 1969), the pH dependence of peaks 2, 3, and 4 is unaffected while that of all other resonances is altered (Table I). Of this second group, only resonance 1 can be clearly observed. At low pH it is downfield from its position in the zinc enzyme, and it moves further downfield, broadening and disappearing, as pH approaches 7 (Pesando, 1973). Above this pH it appears upfield, and its behavior becomes similar to that observed for it in the zinc enzyme. While the chemical shifts of the remaining peaks do not exhibit regular patterns with pH, these resonances also reflect an alteration in the environment of their parent residues near pH 7.

METAL-FREE ENZYME. Nine peaks are observed to low field in the spectrum of the metal-free enzyme (Figure 5 and Table I), two more than in the zinc enzyme, and they possess comparable areas, chemical shifts, and T_1 values. Addition of a full equivalent of zinc to the apoenzyme at high pH causes the spectrum to revert to that of the native zinc enzyme, confirming the absence of paramagnetic impurities in samples of the latter prepared by the regular method.

Assignment of peaks in the apoenzyme is difficult since the zinc and metal-free states interconvert slowly on the pmr time scale. Resonances 2, 3, 4, and 7 of the native enzyme appear to be essentially unaltered while the pH dependence and/or chemical shift of peaks 1, 5, and 6 has been changed. Identification of peak 5 is assisted by its unique chemical shift and the pH dependence of its continued sensitivity to the presence of Fremy's salt. The chemical shift of this resonance no longer reflects a titratable group and corresponds to that observed for it in the low pH form of the zinc enzyme. At high pH it moves slightly upfield and broadens. Peaks 1 and 6 have disappeared and probably correspond to two of the four additional resonances observed (1′, 8′, 9, and 10). Peak 1′ may correspond to 1 in the zinc enzyme since their chemical shifts are in the same region of the spectra, both are selectively broadened in the presence of Fremy's salt, and their respective pH dependencies resemble those of resonances 5′ and 5. Resonances 1′ and 8′ merge above pH 7 as they move upfield, and their behavior is difficult to resolve.

Peaks 9 and 10 seem to reflect groups having pK values of 5.70 and 4.9, respectively. They may represent residues located near positively charged groups on the protein or simply reflect local unfolding of the enzyme at low pH. Both peaks are broadened in the presence of Fremy's salt, as are 7′ and 8′. Anion or sulfonamide inhibitors do not affect these spectra.

CADMIUM CARBONIC ANHYDRASE. Eight resonances

[3] All previous experiments were conducted at an ionic strength of between 0.03 and 0.05 using sodium sulfate as the supporting electrolyte, but it was necessary to raise this value to 0.13 in the chloride study to ensure meaningful results. To investigate the effects of changes in ionic strength on the spectrum, the present data for the free enzyme at an ionic strength of 0.03 were compared with those of Cohen *et al.* (1972) who conducted their experiments at an ionic strength of 0.3, again employing the noninhibitory sulfate anion (Table II of preceding paper). The pK of the group giving rise to peak 5 is unchanged at 8.2 in both studies while the data for peak 4 can only be fitted by a theoretical titration curve at the higher ionic strength. Since the behavior of the group reflected by resonance 4 changes comparably in the presence of high concentrations of both chloride and sulfate, it appears to be a noninhibitory effect. The effect of chloride on the pK value of the residue reflected by peak 5 is specific for the inhibitory anion. Cohen *et al.* (1972) observed resonance 1 only over a narrow low pH range where its chemical shift is independent of pH.

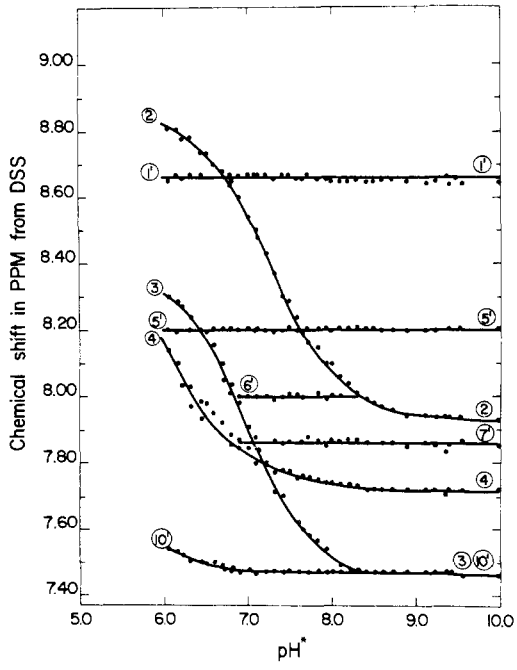

FIGURE 6: pH dependence of resonances in the low field aromatic region of the pmr spectrum of cadmium HCA-B.

are observed in the low-field region of the spectrum of the cadmium enzyme (Figure 6), and they possess comparable areas, line widths, and T_1 values. Assignment of peaks is facilitated by the near identity of the spectra of the Zn(II) and Cd(II) enzymes (Figure 6 and Table I, and Figure 2 of preceding paper). The most significant difference is that the chemical shifts of peaks 1 and 5 are now independent of pH. Both peaks are farther downfield than in the metal-free enzyme but not so far as in the zinc enzyme at high pH. The behavior of peaks 2 and 3 is unaltered while that of 4, 6, and 7 is only slightly affected. A fourth pH dependent resonance, apparently identical with number 10 of the apoenzyme, appears to high field. Metal-free and cadmium states of the enzyme interconvert slowly on the pmr time scale.

COBALT CARBONIC ANHYDRASE. The pmr spectrum of cobalt substituted carbonic anhydrase differs markedly from those of the zinc and metal-free enzymes with broadening of all signals in the aromatic region and the appearance of a large new aromatic peak centered at 6.3 ppm from sodium 2,2-dimethyl-2-silapentane-5-sulfonate. While Co(II) exchanges slowly with the apoenzyme on the pmr time scale, the dissociation of an appreciable fraction of the metal from the active site presumably accounts for the general broadening of all resonances. The chemical shift and pH dependence of one of the peaks in the histidine region resemble those of peak 3 of the zinc enzyme. Acetazolamide has little effect on the spectrum from 7 to 9 ppm while Zn(II) causes a slow loss of the characteristic reddish-blue color of the cobalt enzyme and the appearance of a pmr spectrum similar to that of the zinc enzyme but devoid of the usual fine structure. Only peak 1 is visible to low field, having been unobservable in the cobalt enzyme. Presumably the remaining resonances are broadened by dipolar interactions with free cobalt in solution.

Discussion

Residues Near the Metal Ion. EFFECT OF INHIBITORS. Azide and cyanide are strong inhibitors of carbonic anhydrase that appear to bind directly to Zn(II) (Coleman, 1967; Riepe and Wang, 1968) without producing significant conformational changes in the enzyme (Lindskog *et al.*, 1971; Kannan *et al.*, 1971; Coleman, 1965). Resonances whose magnetic environments change on addition of these inhibitors should reflect residues at the active site, although nonspecific interactions with the enzyme may also be recorded. Aromatic sulfonamides such as acetazolamide are more potent and very specific inhibitors of carbonic anhydrase that also bind directly to the metal ion (Kannan *et al.*, 1971; Lindskog, 1963, 1966; Fridborg *et al.*, 1967; Fabry *et al.*, 1970) with minimal conformational changes in the enzyme (Coleman, 1965; Liljas, 1971; following paper). Their use should also permit identification of resonances of residues at the active site, including ones not detected with the smaller anion inhibitors. Since interaction of azide and cyanide with HCA-B is rapid on the pmr time scale, the behavior of affected resonances can be determined in both the free and inhibited states and assists in interpreting spectra obtained with the slowly exchanging sulfonamides. Results of studies with these inhibitors (Figures 1–3) indicate that peaks 2, 3, and 4 are unaffected by inhibition of the enzyme while the chemical shifts and/or pH dependencies of peaks 1 and 5 are changed in all instances, implying that the latter but not the former resonances reflect groups located at the active site.[4] The residues giving rise to peaks 6 and 7 likewise appear to be located at the active site, with the latter resonance being most sensitive to the presence of the larger sulfonamides (following paper).

MODIFICATIONS OF THE ACTIVE SITE. Residues bound to or near Zn(II) should have their magnetic environments selectively altered on removal or replacement of the metal ion, providing specific and highly sensitive methods for determining their corresponding resonances. While the metal-free enzyme is catalytically inactive and does not bind inhibitors, crystallographic (Kannan *et al.*, 1971; Liljas *et al.*, 1972; Liljas, 1971), immunologic (Wistrand and Rao, 1968), and optical rotatory dispersion studies (ORD) (Coleman, 1965) reveal that it retains its original conformation. While the chemistry of cadmium is similar to that of zinc, substitution of Cd(II) for Zn(II) also yields a catalytically inactive enzyme (Lindskog and Malmstrom, 1962) that does not bind sulfonamide inhibitors (Coleman, 1965, 1967) but appears to retain its original conformation (Lindskog *et al.*, 1971; Kannan *et al.*, 1971; Coleman, 1967). Pmr studies with both modified enzymes (Figures 5 and 6) indicate that peaks 1, 5, and 6 reflect residues close to the metal while peaks 2, 3, 4, and 7 do not. Although cobalt carbonic anhydrase closely resembles the native enzyme in its catalytic activity, conformation, and ability to bind anion and sulfonamide inhibitors (Coleman, 1967; Lindskog and Malmstrom, 1962; Lindskog *et al.*, 1971), substitution of paramagnetic Co(II) for Zn(II) alters the magnetic environments of so many residues that those at the active site cannot be selectively identified. Carboxymethylation of histidine-200 at the active site confirms the above assignments. Changes in the pmr spectrum of this weakly active enzyme appear to result from direct interaction of the parent residues of affected resonances with the new carboxy-

[4] The distinctive spectra produced by azide and cyanide may indicate that these molecules stabilize slightly different conformations of the inhibited enzyme. Differences in orientation of the bound anions and existence of unequal magnetic fields near them may explain their unique effects on the direction and magnitude of the changes in chemical shift of peaks 1 and 5.

methyl group. ORD studies suggest that the carboxymethylated enzyme retains its original conformation (Bradbury, 1969).

PEAKS REFLECTING THE SAME RESIDUE. Identity of the pK values of the groups they reflect, location of their imidazole side chains at the active site, and failure of their parent residues to titrate in any of the inactive states of the enzyme suggest that resonances 1 and 5 may represent the C-2 and C-4 protons of a single histidine. The occurrence of the half-maximal changes in their chemical shifts on addition of azide at the same ratio of inhibitor to enzyme and the comparable increase in the pK values of their parent residues in the presence of chloride (Figure 4, Table I) further support this assignment. This would mean that only four of the eleven histidines of HCA-B are observed to titrate in the active enzyme, in agreement with results of the potentiometric determinations (Riddiford, 1964). Location of resonances 1 and 5 downfield from the values of chemical shift normally expected for histidine C-2 and C-4 protons may reflect their proximity to deshielding magnetic fields induced in one or more of the nearby histidines at the active site (Kannan *et al.*, 1971; Liljas *et al.*, 1972). In the remainder of this discussion it will be assumed that peaks 1 and 5 arise from a single histidine.

Nature of the Group Controlling Catalytic Activity. CHEMICAL IDENTITY. The histidines reflected by resonances 1, 2, 3, and 5 of HCA-B all possess pK values that warrant their consideration for assignment to the group controlling catalytic activity. However, only the residue reflected by peaks 1 and 5 appears to be located at the active site. Its pK of 8.24 compares favorably to the value obtained for this group in HCA-B by other techniques (Coleman, 1967; Fabry *et al.*, 1970; Ward, 1970), although reported values range from 7.3 to 8.2 (Pesando, 1973). Addition of anions or sulfonamides, removal of zinc, or substitution of cadmium for zinc at the active site, procedures known to abolish enzymatic activity (Lindskog *et al.*, 1971; Coleman, 1967, 1971; Lindskog and Malmstrom, 1962), prevent titration of this residue. Partial inhibition of carbonic anhydrase by anions shifts the apparent pK value of the group controlling catalytic activity to higher values as anions appear to compete with the latter for the metal (Lindskog, 1966), and a similar selective effect is observed on the titration curves reflected by resonances 1 and 5 in the presence of chloride (Figure 4). (Alternatively, peaks 1 and 5 may represent a histidine residue at the active site that merely reflects titration of the group controlling catalytic activity. Although the precise mechanism(s) responsible for the observed changes in chemical shift of peaks 1 and 5 with pH in this scheme is not readily apparent, such a possibility is consistent with the present data and cannot be excluded at this time.)

REVERSIBLE BINDING TO ZINC. The downfield movement of peaks 1 and 5 with increasing pH in the native enzyme requires explanation since the C-2 and C-4 protons of histidine and imidazole usually move upfield and demonstrate a fourfold greater change in chemical shift under comparable conditions. Hydrogen bonding, perturbation by induced local magnetic fields, and electrostatic interactions can produce downfield changes in chemical shift of proton resonances in diamagnetic proteins. However, histidine C-2 and C-4 protons are unlikely to be directly involved in formation of hydrogen bonds, and it is improbable that variations in the small magnetic fields induced in neighboring chemical groups, as with local conformational changes, would produce similar effects on the resonances of protons 0.425 nm apart. The C-2 and C-4 proton resonances of imidazole also move downfield upon formation of complexes with metal ions, a process favored at high pH, though the magnitude of this change is less than that produced by protonation. In a 4:1 complex of imidazole with Zn(II) in dimethyl sulfoxide, Wang and Li (1966) report downfield changes in chemical shift for the C-2 and equivalent C-4, 5 protons of imidazole that are equal to those observed here for resonances 5 and 1, respectively. By analogy, the changes in chemical shift observed for peaks 1 and 5 of HCA-B with increasing pH might result from reversible formation of a complex between histidine and zinc at the active site.[5] Experiments with the catalytically inactive yet conformationally unaltered (Lindskog *et al.*, 1971; Coleman, 1965, 1967; Lindskog and Malmstrom, 1962) metal-free and cadmium-substituted carbonic anhydrases support this proposal. In the metal-free enzyme, the chemical shift of resonance 5 does not reflect a titratable residue and corresponds to that observed for this peak in the low pH form of the zinc enzyme.[6] (Resonance 1 of the zinc enzyme cannot be definitively assigned to a peak in the metal-free enzyme.) In cadmium carbonic anhydrase the histidine giving rise to resonances 1 and 5 again fails to titrate. The chemical shifts of these peaks are farther downfield than in the metal-free enzyme but not so far as in the zinc enzyme at high pH, where this exchangeable histidine ligand is thought to be bound to the metal. Pmr studies of imidazole-metal complexes (Wang and Li, 1966; Carlson and Brown, 1966) demonstrate that the magnitude of the downfield change in chemical shift of the ring protons of the ligand increases with increasing strength of the metal-ligand bond and confirm that Zn(II) interacts more strongly with imidazole than does Cd(II) (Carlson and Brown, 1966). With its larger ionic radius (0.92 Å *vs.* 0.69 Å) cadmium may bind to the exchangeable histidine residue over the entire pH range studied.

Proposed Catalytic Mechanisms. BINDING OF AN IONIZABLE HISTIDINE TO ZINC. The pH dependent coordination of the histidine reflected by peaks 1 and 5 to the metal ion in HCA-B appears to be intimately associated with ionization of the group controlling enzymatic activity. This ionization may represent loss of the pyrrole proton of this neutral ligand when it binds to Zn(II), forming an imidazolate anion and juxtaposing a strong base and a powerful Lewis acid at the active site. This histidine, when bound to zinc as an anion, may act as a general base catalyst in the hydration of carbon dioxide and be replaced as a metal lig-

[5] Since ligands to the metal are in rigid environments, their correlation times should approximate that of the protein, raising the question of whether or not their resonances are too broad to be observed. Calculation of the spin–spin (T_2) relaxation time for an imidazole C-2 proton interacting with its C-4 neighbor and possessing the correlation time of 1×10^{-8} sec (Chen and Kernohan, 1967) of carbonic anhydrase yields an approximate value of 2.2 sec. The predicted line width of approximately 0.15 Hz ($T_2 = 1/\pi\Delta\nu_{1/2}$) for such a resonance therefore does not *a priori* exclude its ready detection in these studies. Observation of the resonances of the C-2 protons of all 11 histidines is not necessarily expected, however, since variation in interproton distances will also affect relative line widths.

[6] In addition to whatever role it serves in catalysis, Zn(II) may be involved in maintaining the structure of the protein at the active site. Removal of the metal ion may cause a partial collapse of protein structure so that this histidine is no longer able to titrate. Observation (Lindskog, 1963) of an increase in the number of protons liberated by addition of Zn(II) to the apoenzyme at high pH supports this proposal.

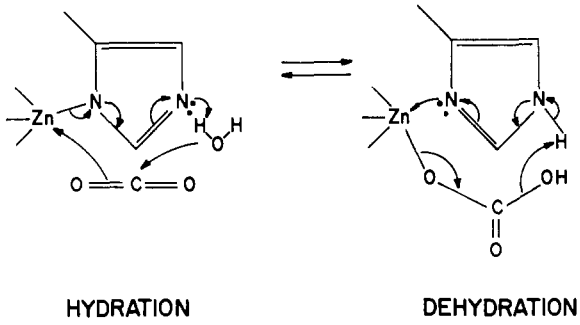

FIGURE 7: Schematic representation of the proposed model for the catalytic mechanism of carbonic anhydrase. The histidine ligand on zinc exists as an imidazolate anion. Net charges are not indicated since the formal charge on the metal is not known. Arrows indicate the direction of the movement of electrons.

and by an oxygen of the substrate in the course of the reaction (Figure 7). This concerted mechanism allows Zn(II) to serve as a Lewis acid by coordinating to the incipient bicarbonate ion, as well as promoting the general base catalysis by imidazole. The histidine–metal complex probably exists in a strained configuration in the active enzyme so that its imidazole–zinc bond is labile and readily broken on addition of substrates or inhibitors. Monovalent anions could compete with the exchangeable histidine ligand for a site on the metal at low pH, displacing the equilibrium for generation of the active enzyme. On binding to the high pH form of the enzyme, sulfonamides may displace and protonate the exchangeable histidine, in agreement with the model of Taylor *et al.* (1970). Orientation of substrates may be facilitated by hydrogen bonds to water, or to serine or threonine, as discussed in the following paper.

Construction of the reaction system depicted in Figure 7 using space filling models confirms that a strained configuration for the imidazole–metal bond is necessary to meet the steric requirements of a reaction involving a single water molecule. Substrates must approach the metal ion from beneath the plane of the imidazole ring, and bent hydrogen bonds are needed. Wang (1970) has proposed that such strained hydrogen bonds may play important roles in enzymatic catalysis by permitting more rapid transfer of protons than unstrained ones. However, the number of water molecules in the proton transfer reaction is not known, and more than one might well be involved. For example, the reactive water molecule adjacent to the imidazole group in the hydration reaction might ionize and attack an additional water molecule which in turn reacts with the substrate.

IONIZABLE WATER LIGAND. The ionizable group controlling catalytic activity in carbonic anhydrase appears to be bound to the metal ion at high pH (Lindskog *et al.*, 1971; Coleman, 1967; Taylor *et al.*, 1970; Fabry *et al.*, 1970), leaving only three histidines and a water molecule to be considered for this role (Kannan *et al.*, 1971; Liljas *et al.*, 1972). A histidine ligand might function in catalysis as indicated above, while it has been proposed by several laboratories (Khalifah, 1971; Coleman, 1967; Bradbury, 1969; Lindskog and Coleman, 1973; Riepe and Wang, 1968) that a water molecule bound to zinc may ionize near pH 7 to generate an hydroxide ion that serves as an attacking species in the hydration reaction. Potentiometric experiments with free imidazole (Martin and Edsall, 1960; Bauman and Wang, 1964; Brooks and Davidson, 1960; George *et al.*, 1964) support the theoretical proposal (Coryell and Pauling, 1940; Martell and Calvin, 1952) that the pK value of 14.2 for this group can be reduced by approximately 7 pH units on formation of complexes with divalent transition metal cations. Such a change would give it an appropriate pK value for the group controlling enzymatic activity. Although the pK for ionization of water is reported to be reduced from 15.74 to between 9 and 10 on binding to Zn(II), correction for the presence of six water molecules in the aqueous complexes studied increases this value by 0.8 unit (Prince and Woolley, 1972) to between 9.8 and 10.8. While the pK of metal-bound water appears to be too high for the group controlling enzymatic activity, pK values of both types of metal ligands might be further reduced by additional forces acting at the active site.

The low nucleophilicity of its conjugate base also argues against an ionizable water molecule bound to Zn(II) controlling carbonic anhydrase activity (Wang, 1970). While the basicity and nucleophilicity of the imidazolate anion are also reduced when it is bound to Zn(II), an anionic histidine ligand would also be fixed by the protein so that its bond to the metal ion might be strained and readily broken on addition of substrates. This strain might be angular and/or radial in nature and could derive from the rigid antiparallel β pleated sheet structure that gives rise to all three histidine ligands to the metal. This proposed mechanism assigns to the protein the role of creating a highly distorted environment at the zinc ion and supports the entatic or strain model for the activity of metalloenzymes (Vallee and Williams, 1968; Williams, 1971). In contrast, the ionizable water theory suffers from the weakness of assigning no role to this 30,000 molecular weight protein save that of enforcing an imperfect substrate specificity. Constrained binding of a ligand from the protein to the metal has also been proposed by Dennard and Williams (1966) to explain the distortion of the coordination geometry of Co(II) that occurs in this metal-substituted enzyme at high pH but not in the presence of inhibitors.

Assignment of Peaks to Specific Residues in the Primary Sequence. Availability of the primary sequences of HCA-B (Andersson *et al.*, 1972; Lin and Deutsch, 1973) and HCA-C (Henderson *et al.*, 1973; Lin and Deutsch, 1974; Laurent-Tabusse *et al.*, 1972) and the knowledge of the three-dimensional structure of HCA-C (Kannan *et al.*, 1971; Liljas *et al.*, 1972) permit tentative assignment of the observed resonances to specific residues. Six of the eleven histidines of HCA-B are thought to be located within the cavity forming its active site, and the remaining five are scattered throughout the protein. Of the second group, residues 107 and 122 appear to be buried within the protein where they may not titrate and where proximity to neighboring groups may sufficiently broaden their resonances that they cannot be observed. Residues 40, 103, and 243, the remaining members of the second group, appear to be located on the surface of the protein and may give rise to peaks 2, 3, and 4. Since resonance 3 seems to be the only one unaffected in the cobalt enzyme, it may reflect the residue farthest from the metal ion, which appears to be number 40.

Resonances 1 and 5 reflect the ring protons of one of the three histidine ligands to the metal, possibly number 119, since this residue is coordinated through its N-1 rather than its N-3 nitrogen. Resonance 6 may be that of a ring proton of one of the two remaining ligands, numbers 94 and 96. Resonance 7 reflects one of the three remaining histidines at the active site, number 64, 67, or 200.

Addendum

Prior to submission of this manuscript, Appleton and Sarkar (1974) described studies of the ionization of water and of the pyrrole N–H of histidine in zinc chelates serving as models for the active site of carbonic anhydrase. These authors report a pK of 7.0–7.3 for the pyrrole N–H of histidine and of 9.12 for metal-bound water. In systems in which both ionizations can occur, ionization of the pyrrole hydrogen takes precedence.

Figures depicting the pH dependence of resonances of the carboxymethylated enzyme and the effect of Zn(II) on the apoenzyme were submitted to the reviewers for their examination but were omitted from the final text to conserve space. Copies will be furnished by the author to the interested reader.

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