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Proton Magnetic Resonance Studies of Carbonic Anhydrase. I. Identification of Histidine Resonances†

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ABSTRACT: Nuclear magnetic resonance (nmr) spectra of human carbonic anhydrase B recorded in deuterium oxide reveal seven discrete single proton resonances between 7 and 9 ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Simplification of spectra by use of Fremy's salt, comparison of peak widths at intersections, and evaluation of the results of inhibition and modification experiments permit determination of the pH dependencies of these resonances. Five of these peaks change position with increasing pH; three move upfield by approximately 95 Hz and two move downfield by 10 and 23 Hz. The first three reflect residues with pK values of 7.23, 6.98, and 6 and can be assigned to the C-2 protons of histidines. The two remaining pH dependent resonances reflect groups with pK values of 8.2 and 8.24. Their line widths and T_1 values are comparable to those of the first group, and they also appear to reflect C–H protons of histidines. Despite the structural and functional similarities of the B and C isozymes of human carbonic anhydrase, few of the low field resonances appear to be common to both. Six histidine C-2 protons are observed in the C enzyme and reflect groups with pK values of approximately 7.3, 6.5, 5.7, 6.6, 6.6, and 6.4. A seventh peak contains two protons and moves upfield with increasing pH without titrating. A final resonance to low field moves downfield with increasing pH and reflects a group with a pK between 6 and 7. Its behavior resembles that of peak 1 of the human B enzyme, and it also appears to be a histidine C–H proton. This peak may reflect a conserved residue in the two isozymes that plays an important role in enzymatic function, as discussed in the following paper.

Carbonic anhydrase is a zinc metalloenzyme of approximately 29,000 molecular weight found throughout the plant and animal kingdoms where it catalyzes the reversible hydration of carbon dioxide. The enzyme consists of a single polypeptide chain with one tightly bound Zn(II) (Lindskog and Malmstrom, 1962) that is essential for catalytic activity and binding of anion and sulfonamide inhibitors. Zn(II) can be replaced by a number of divalent transition metal cations without changing the conformation of the enzyme, but only the cobalt derivative is catalytically active (Linkskog and Malmstrom, 1962; Linkskog *et al.*, 1971; Coleman, 1967). Two major forms of carbonic anhydrase in man and other higher organisms (Nyman, 1961; Rickli *et al.*, 1964) have different activities and are designated the B and C isozymes. They are thought to possess similar three-dimensional structures (Lindskog *et al.*, 1971; Andersson *et al.*, 1972; Coleman, 1971) and active sites (Lindskog and Nyman, 1964; Lindskog *et al.*, 1971; Khalifah, 1971). They demonstrate comparable binding strengths for Zn(II) (Lindskog and Nyman, 1964) and for inhibitors (Taylor *et al.*, 1970; Verpoorte *et al.*, 1967), and the general features of the visible absorption spectra of their cobalt derivatives are nearly identical in the free and inhibited states (Lindskog and Nyman, 1964; Taylor *et al.*, 1970). Both enzymes also catalyze hydration of certain aldehydes (Pocker and Meany, 1965, 1967) and hydrolysis of various esters (Verpoorte *et al.*, 1967; Pocker and Stone, 1967), though at much lower rates.

The pH dependencies of the turnover number of carbonic anhydrase and of the visible absorption spectrum of its cobalt derivative, both of which describe sigmoidal curves with points of inflection near neutrality, are thought to reflect ionization of a group on or near the metal ion controlling catalytic activity (Khalifah, 1971; Kernohan, 1964). A 2-Å crystallographic study of the human C enzyme shows

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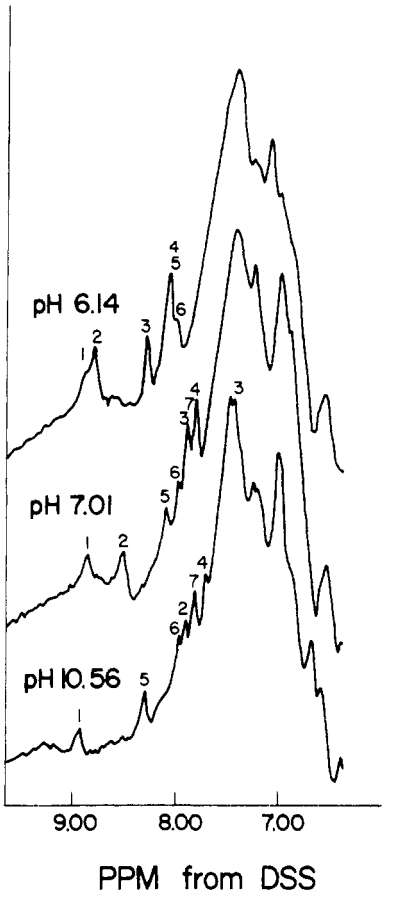

FIGURE 1: Aromatic region of the proton magnetic resonance spectrum of HCA-B at various pH values. Peaks are numbered from low field at pH 6. The HDO signal is to high field and pH values are uncorrected meter readings.

Zn(II) to be located at the bottom of a conical cavity forming the active site with three histidines and a water molecule serving as its ligands (Kannan *et al.*, 1971; Liljas *et al.*, 1972). Amino acid modifications confirm the presence of additional histidines at the active sites of both enzymes, giving a total of four such residues in the C enzyme and six in the B (Liljas *et al.*, 1972).

This paper discusses the pH dependence and chemical identity of resonances in the histidine region of the proton magnetic resonance (pmr) spectrum of the human B and C enzymes (HCA-B and HCA-C).[1] In the following two papers these resonances are used as probes to identify the group controlling catalytic activity and to study enzyme-inhibitor complexes in HCA-B.

Experimental Section

Materials. Packed human erythrocytes were kindly provided by Dr. C. Erich and Ms. C. Hallett of the New York City Blood Bank. All chemicals were reagent grade or better unless otherwise indicated and were used without further purification. Acetazolamide (2-acetamido-1,3,4-thiadiazole-5-sulfonamide) was a gift from Dr. J. M. Smith at American Cyanamide and ethoxzolamide (6-ethoxy-2-benzothiazolesulfonamide) was provided by Marvin Guthaus at the Upjohn Company. Pmr spectra and melting points of these two drugs confirmed their purity.

Deionized water was used throughout these experiments and all glassware used after the initial purification steps was cleaned in acid. Where possible, polyethylene materials were used and cleaned in like fashion. Dialysis tubing was freed of metal ions before use.

Preparation of Enzyme. Carbonic anhydrase was purified by the chloroform–ethanol method of Rickli *et al.* (1964) as adapted by Armstrong *et al.* (1966). Purity of the enzyme was determined by titrating esterase activity against increasing concentration of ethoxzolamide using *p*-nitrophenyl acetate as substrate (Maren *et al.*, 1960). Enzyme assayed in this fashion was typically between 95 and 100% active. Optical densities of 0.1% solutions of the B and C enzymes were taken as 1.63 and 1.87, respectively (Armstrong *et al.*, 1966).

Samples for Pmr Spectroscopy. Samples were prepared using the protein as buffer and 0.01 M sodium sulfate as the supporting electrolyte and dialyzed against several changes of the latter to remove exchangeable protons. All solutions of carbonic anhydrase were from 2.4 to 3.2 mM in protein as determined spectrophotometrically. Samples were prepared for immediate use from stock solutions at high and low pH* (hereafter simply referred to as pH), by mixing aliquots of the two to achieve any desired pH value.

pH measurements are given as uncorrected meter readings, and pK values were calculated directly from them since the deuterium isotope effects on the glass electrode pH readings and on the dissociation constant of histidine have been reported to be equal and opposite (Roberts *et al.*, 1969; Sachs *et al.*, 1971). pH readings for each sample were determined before and after obtaining its spectrum; spectra were discarded if the two values differed by more than 0.03 pH unit.

Recording of Spectra. Spectra were recorded using a JEOLCO 100 MHz spectrometer converted to Fourier transform operation by Drs. A. G. Redfield and R. K. Gupta (Redfield and Gupta, 1971a) at the IBM Watson Laboratories, Yorktown Heights, N.Y.; 5-mm nmr tubes with coaxial inserts containing hexafluorobenzene as a lock signal were used throughout. Sample volumes were approximately 0.3 ml. The HDO signal and its side bands were eliminated from spectra by using a long, weak 90° pulse adjusted so that its power spectrum effectively vanished at the HDO frequency (Redfield and Gupta, 1971b). Distortion of spectral intensities caused by use of this long pulse were corrected by computer (Redfield and Gupta, 1971a,b); 1000–1500 free induction decay signals were averaged for each spectrum, and the temperature of the probe was maintained at 23 ± 0.5°.

Results

Resonances to Low Field. Pmr spectra of the aromatic region of HCA-B (Figure 1) reveal seven single proton resonances to low field whose pH dependencies are shown in Figure 2. The number of protons represented by these peaks was estimated from the ratio of their combined areas to that of the large aromatic signal at several pH values, knowing that there are 139 aromatic protons (Armstrong *et al.*, 1966). Since resonances 2, 3, and 4 can be assigned to the C-2 protons of histidines (see below), they provide internal standards for calculating peak areas.

Five of the observed resonances reflect the titration behavior of their parent amino acids, although the data for peak 4 cannot be fitted by a theoretical titration curve. Of these, resonances 2, 3, and 4 are estimated to move upfield

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

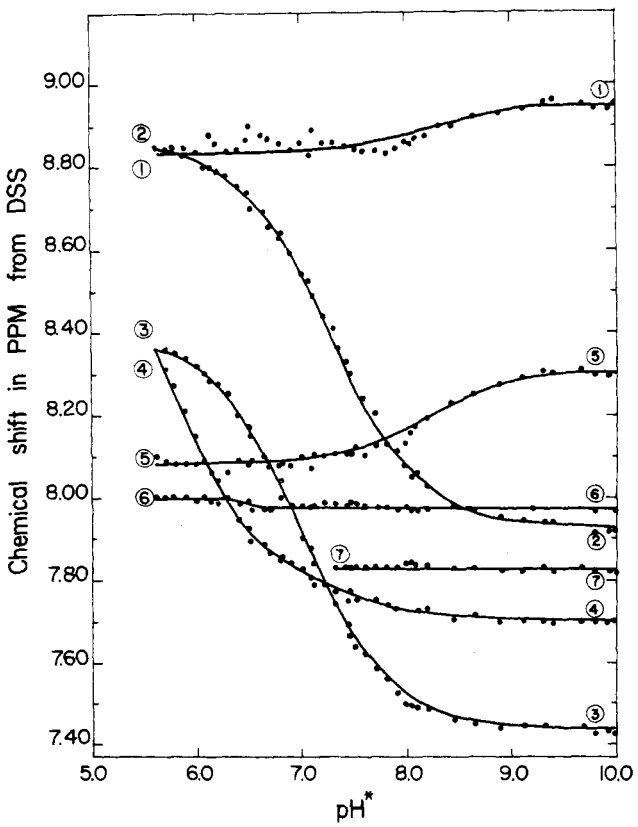

FIGURE 2: pH dependence of the chemical shift of resonances in the histidine region of the pmr spectrum of HCA-B. The numbering scheme and experimental conditions correspond to those of Figure 1. Curves for resonances 1, 2, 3, and 5 were calculated using the Henderson-Hasselbalch equation and represent the best fit to experimental points. The curve describing resonance 4 was drawn by hand. Resonances 6 and 7 represent nontitrating residues. The behavior of individual peaks was determined as described in the text.

Table I: Spin-Lattice Relaxation Times (T_1).

Group	t_{null} (msec)	T_1 (msec)
Aromatic peak of HCA-B	170 ± 10	245 ± 14
Aliphatic peaks of HCA-B	150 ± 20	216 ± 29
Amide peak of HCA-C	100 ± 20	144 ± 29
Low field aromatic protons of HCA-B:		
1	240 ± 20	346 ± 29
2	270 ± 30	390 ± 43
3	270 ± 30	390 ± 43
4	270 ± 30	390 ± 43
5	240 ± 20	346 ± 29
6	180 ± 20	260 ± 29
7	205 ± 45	296 ± 65

by approximately 95 Hz with increasing pH, the first two reflecting groups having pK values of 7.23 and 6.98, respectively. The pK of peak 4 is estimated to be around 6. Peaks 1 and 5 move downfield with increasing pH by 10 and 23 Hz and reflect groups with pK values of 8.2 and 8.24, respectively. Resonance 7 is only visible above pH 7. Broad, rapidly relaxing amide resonances in this region of the spectrum prevent precise calculation of individual spin-lattice relaxation times (T_1 values) from the variation in signal intensity with time following a 180° prepulse, but approximate values are obtained from the time delays producing null point values (Table I).

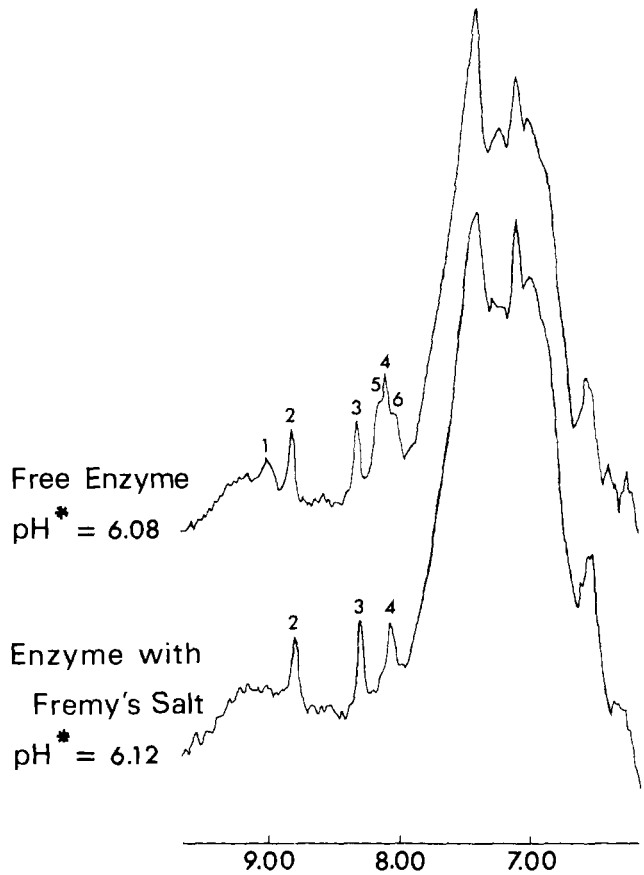

FIGURE 3: Effect of addition of Fremy's salt on the pmr spectrum of the aromatic region of HCA-B. 5 μl of Fremy's salt (dissolved in 0.1 M phosphate buffer (pH 9)) was added to the enzyme to give a final concentration of approximately 0.8 mM. An increase in the pH of the solution caused a small change in the chemical shift of peak 4. In this aged sample, peak 1 is farther downfield than usual, permitting clear observation of the effects of the salt upon it.

Studies with Fremy's Salt. Fremy's salt (potassium nitrosodisulfonate) is a free radical that inhibits HCA-B with a K_I of approximately 50 mM at pH 8. Addition of 1 mol of Fremy's salt to 3 of carbonic anhydrase at low pH broadens resonances 1, 5, 6, and 7 while leaving 2, 3, and 4 unaffected (Figure 3). As pH increases, the missing peaks sharpen and reappear in the order 1, 6, 5, and 7. At constant pH, these peaks broaden without change in chemical shift as the concentration of Fremy's salt increases. Addition of stronger inhibitors only partially reverses these effects. Less than 5% of the enzyme is inhibited at the concentration of Fremy's salt routinely employed in these experiments. Since Fremy's salt is increasingly labile outside the pH range 6-9, samples containing it were only used for a few hours to avoid significant decomposition.

Human Carbonic Anhydrase C. The pmr spectrum of the aromatic region of HCA-C at several pH values is shown in Figure 4. A maximum of eight resonances can be observed to low field, and their pH dependence is shown in Figure 5. Measurements at low pH indicate that single protons account for resonances 1-7. Resonance 8 appears to correspond to two protons over the entire pH range studied. Measurement of spin-lattice relaxation times indicates that

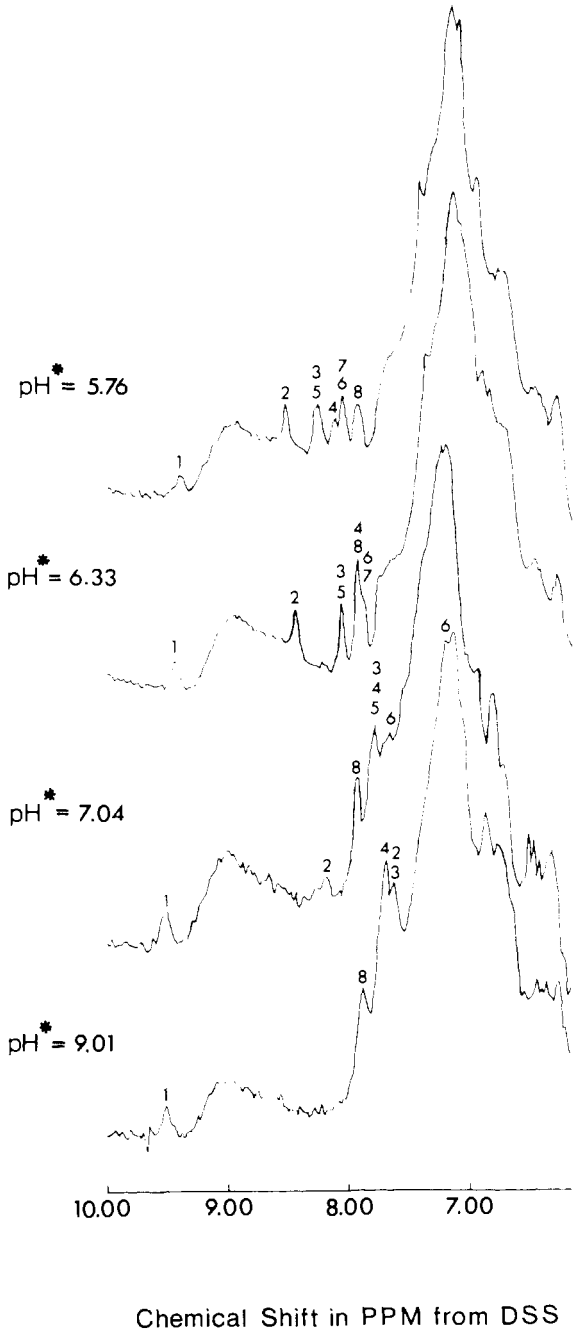

FIGURE 4: Aromatic region of the pmr spectrum of HCA-C at various pH values. Peaks are numbered from low field at pH 5.3.

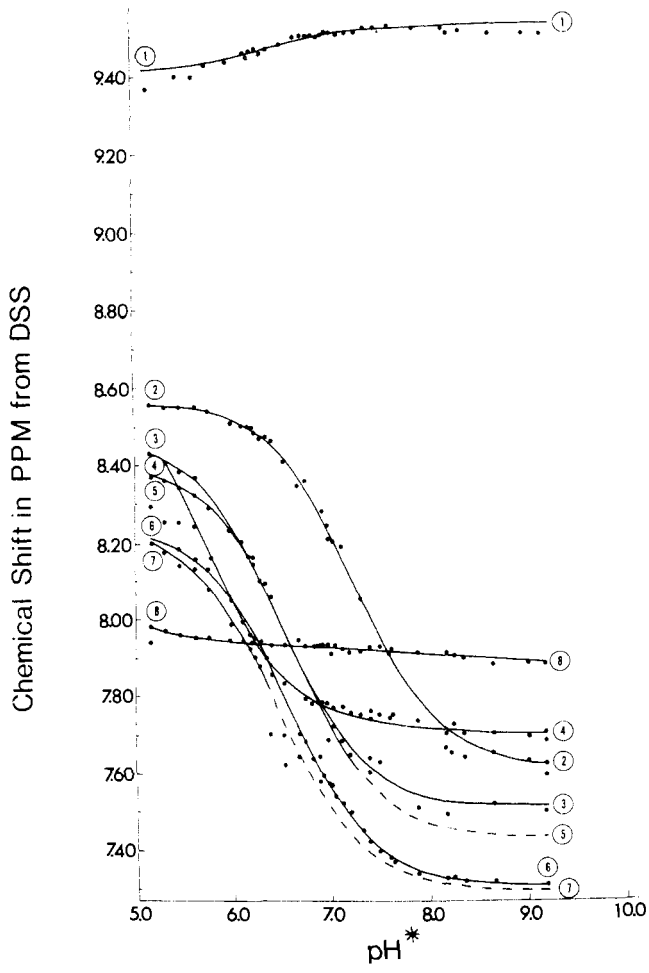

FIGURE 5: pH dependence of resonances in the histidine region of the pmr spectrum of HCA-C. The numbering scheme corresponds to that of Figure 5. Dashed lines represent the theoretical titration curves at high pH of resonances that have broadened.

peaks 1–8 have comparable T_1 values while that of the broad absorption band at low field is much shorter. All data are compared to theoretical titration curves, although not all of the observed resonances fall on or near them. With the exception of peak 1, a maximum pH dependent change in chemical shift of approximately 95 Hz is assumed. That for peak 1 is about 12 Hz. The intensity of the broad absorption band centered at 9 ppm decreases with time and increasing pH in deuterium oxide.

Peak 1 is to extreme low field and moves downfield as pH is increased. The pK value of the group that it reflects is between 6 and 7 but cannot be precisely determined because the enzyme is unstable at low pH and the available data cannot be fitted by a theoretical titration curve. Resonances 4 and 6 are clearly visible over the entire pH range studied and reflect groups titrating with pK values of 5.74 and 6.57, respectively. Peaks 2, 3, 5, and 7 broaden considerably with increasing pH, and their behavior is clearly defined only below pH 7. The best fit of the data to theoretical titration curves gives respective pK values of 7.25, 6.49, 6.63, and 6.43 for their parent residues, but the error for some of these pK values may be considerable. Resonance 8 moves gradually upfield with increasing pH, but the peak does not appear to reflect a titratable residue.

Interaction of HCA-C with acetazolamide is slow on the pmr time scale while that with azide is rapid, allowing resonances to be assigned in the free and inhibited states. Resonance 1 loses its pH dependence in the presence of both inhibitors, moving downfield on addition of azide but not acetazolamide.

Discussion

Identification of Observed Resonances. The pH dependence of individual peaks must be established before their behavior can be analyzed and the chemical nature of their parent residues determined. In complex spectra such as those of Figures 1 and 2 this task is greatly simplified by use of Fremy's salt. This free radical inhibitor interacts rapidly with the enzyme on the pmr time scale and selectively broadens resonances near its site of binding through electron–proton dipolar interactions (Pesando, 1973). Broadening of peaks 1, 5, 6, and 7 by this compound reduces the number of cross-over points in Figure 2 from nine to two to

Table II: Ionization Constants of Histidine Residues Observed by Pmr Spectroscopy in HCA-B.

	Resonances					
	1	2	3	4	5	7
Present study	8.2	7.23	6.98	6[a]	8.24	NO[b]
King and Roberts (1971)	NR[c]	7.23	7.00	6.04	NO	5.91
Cohen *et al.* (1972)	NO	7.23	6.93	6.09	8.2	5.88

[a] Data not fit by a theoretical titration curve. [b] Not observed to titrate. [c] Not reported.

Table III: Comparison of Experimental Conditions in Three Pmr Studies of Carbonic Anhydrase.

	King and Roberts (1971)	Cohen *et al.* (1972)	Present Work
Field strength	100 MHz (cw)	220 MHz (cw)	100 MHz (pulsed)
Protein concn (%)	18	10	5–10
Ionic strength	0.2	0.3	0.03
Temperature (°C)	27.5	22	23
Preparation of enzyme	Ion exchange method[a]	Chloroform–ethanol method[b]	Chloroform–ethanol method[c]

[a] Armstrong *et al.*, 1966. [b] Kandel *et al.*, 1970. [c] Rickli *et al.*, 1964.

permit clear and ready determination of the pH dependence of all seven resonances (Figures 2 and 3).

Peaks 2, 3, and 4 of HCA-B (Figures 1 and 2) can be assigned to the C-2 protons of histidines, in agreement with the results of published studies (Pesando, 1971; King and Roberts, 1971; Pesando, 1972; Cohen *et al.*, 1972; Pesando, 1973). They represent single protons, occur as discrete entities in the low field portion of the pmr spectrum, and reflect titratable residues having pK values of 7.23, 6.98, and 6, respectively. Resonances 1 and 5 possess similar properties, representing residue(s) having respective pK values of 8.2 and 8.24. They differ from peaks 2, 3, and 4 and from those of free histidine in that the changes in their chemical shifts with increasing pH are much smaller and opposite in direction, moving downfield instead of upfield. Resonances 1 and 5 cannot be assigned to other amino acids and may represent the ring protons of histidine(s) in some unusual chemical environment. While peaks 6 and 7 also appear to reflect single protons, they are insufficiently characterized to permit definitive chemical assignment.

Distinguishing between C–H and N–H Protons. Peaks 1–7 are readily distinguished from the resonances of exchangeable protons observed further downfield in aqueous solutions by their line widths and by their slow replacement with deuterium. Peaks 1–6 are all located between 7 and 9 ppm, and their intensities do not vary significantly with changes in time or pH in deuterium oxide solutions (the appearance of peak 7 is pH dependent). In contrast, 11 peaks, each representing a single proton, are observed below 10 ppm in spectra of HCA-B recorded in aqueous solutions (Gupta and Pesando, in press), but none of these peaks is observed in freshly prepared samples in deuterium oxide at any pH. The line widths measured at half-height for the resonances of the exchangeable protons at low field are all greater than 15 Hz while those of peaks 1–7 vary from 5 to 8 Hz.

The spin–lattice relaxation of resonances 1–5 are of comparable length and are substantially longer than those of the remaining aromatic and aliphatic protons of this protein (Table I), suggesting that peaks 1 and 5 as well as peaks 2, 3, and 4 may reflect ring C–H protons of histidines. The T_1 value of resonance 7 at high pH supports a similar assignment while that of resonance 6 is shorter and does not assist in its identification.

Comparison with the Results of Other Investigators. In pmr studies of the aromatic region of HCA-B, both King and Roberts (1971) and Cohen *et al.* (1972) observed four resonances that move upfield with increasing pH and assigned them to the four titratable histidines postulated by Riddiford (1964) to explain the results of potentiometric experiments. Only three such resonances are observed in the present work (Pesando, 1971, 1972, 1973). Resonances 2 and 3 and the pK values of the histidines that they reflect are comparable in all three studies (Tables II and III). Resonance 4 is likewise comparable, but its sensitivity to neighboring charged groups appears to decrease with increasing ionic strength, as discussed in the following paper (Pesando, 1975). The behavior of resonance 7 at low pH is in dispute (Figures 1 and 2). Both Kind and Roberts (1971) and Cohen *et al.* (1972) reported that this peak reflects a titratable residue, the former workers arguing that this resonance overlaps peak 4 below pH 6.5 and the latter arguing that it broadens instead. In the present studies (Pesando, 1971, 1972, 1973; 1975; Pesando and Grollman, 1975), the chemical shift observed for resonance 7 appears to be virtually independent of pH, arguing against its overlap with the peaks of resonances 3 or 4. It seems to broaden and disappear below pH 7.

King and Roberts (1971) did not report observation of pH dependent resonances corresponding to numbers 1 and 5 of Figures 1 and 2, though resonance 5 may correspond to one of the listings in their table of nontitrating peaks. Cohen *et al.* (1972) observed both resonances but reported the presence of peak 1 only over a narrow low pH range where it is independent of pH.

Studies with Fremy's Salt. The selective broadening of peaks 1, 5, 6, and 7 by Fremy's salt confirms the assignment of these resonances to residues at the active site made by inhibition and modification experiments (Pesando, 1975). However, Zn(II) is not required for binding of Fremy's salt at the active site as demonstrated by failure of strong anion and sulfonamide inhibitors to fully reverse the effects of this free radical on the above resonances and by the ability of Fremy's salt to selectively broaden all peaks save 2, 3, and 4 in the metal-free enzyme. Fremy's salt therefore provides a useful means of identifying resonances of residues at the active site in inactive but conformationally unaltered forms of HCA-B (Pesando, 1975). Reappearance of broadened resonances at high pH with Fremy's salt probably reflects weaker binding of the anion to the enzyme following deprotonation of a group at its binding site(s).

Table IV: Ionization Constants of Histidine Residues Observed by Pmr Spectroscopy in HCA-C.

	Resonances							
	1	2	3	4	5	6	7	9
Present study	6.5	7.25	6.49	5.74	6.63	6.57	6.43	NO[a]
Cohen *et al.* (1972)	NO	7.28	6.63	6.10	6.20	7.20	5.96	5.87

[a] Not observed.

If there is only one binding site for Fremy's salt on HCA-B, then the order of reappearance of these missing resonances should reflect their relative distances from the free radical, and their line widths are given by

$$\Delta\nu_{obsd} = \Delta\nu_{free} + F(\Delta\nu_{complex} - \Delta\nu_{free})$$

where $\Delta\nu$ is the line width at half-height of the proton resonances of the protein in the spectra of the observed, free, and complexed states of the enzyme. F is the fraction of complexed enzyme. At a given pH, F will be constant, as will $\Delta\nu_{free}$ and $\Delta\nu_{complex}$ for a given resonance of the protein, thereby determining the value of $\Delta\nu_{obsd}$ for that resonance. The value of $\Delta\nu_{complex}$ for a given resonance increases as the distance of its proton from the nitroso group decreases so that smaller values of F, and therefore higher pH values, are needed to produce a given observed line width. Distances of the parent residues of the broadened peaks from the site of binding of Fremy's salt appear to increase in the order 7, 5, 6, and 1. Insufficient Fremy's salt is present in these experiments to produce significant oxidation of tyrosine (Pesando, 1973).

Human Carbonic Anhydrase C. If histidines play an important role in the catalytic function of carbonic anhydrase, then similar residues would be expected in the B and C isozymes, though their proton resonances need not be detectable by pmr spectroscopy. Peak 1 of HCA-C resembles peak 1 of HCA-B (Figures 1, 2, 4, and 5). It too is located in the extreme downfield region of the spectrum, moves downfield with increasing pH in the free enzyme, and is independent of pH in the presence of inhibitors. The pK of between 6 and 7 reflected by this peak is comparable to that reported for the group controlling catalytic activity in the C-type enzyme. On the basis of its chemical shift, area, pH dependence, line width, T_1 value, and sensitivity to inhibitors, this resonance is assigned to the ring proton of a histidine at the active site of the enzyme. This residue appears to be one of the few histidines conserved in the two human isozymes (Lindskog *et al.*, 1971; Lin and Deutsch, 1974) and may be essential for catalysis. A similar resonance is also observed in the bovine enzyme. Peak 5 is not observed in either of the C-type carbonic anhydrases.

The present pmr study of HCA-C is compared with that of Cohen *et al.* (1972) in Tables III and IV. Differences in reported pK values might reflect the greater ionic strength employed by Cohen *et al.* and the ambiguities inherent in fitting curves to resonances that overlap so greatly. A pH dependent resonance corresponding to peak 8 was observed by Cohen *et al.* (1972), but resonance 1 was not reported, apparently because the region of the spectrum in which it appears was not studied. Dilute phosphate buffer was employed in the present work before its weak inhibitory action was noted (Khalifah, 1971), and it may have altered the spectra of the enzyme to some extent. However, only small changes are observed in the spectrum of HCA-B under these conditions where the inhibitory effects of phosphate are reported to be considerably greater than in HCA-C (Christiansen and Magid, 1970). The observation and behavior of peak 1 are not dependent on the presence of phosphate.

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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.