Carbon-13 Nuclear Magnetic Resonance Probe of Active-Site Ionizations in Human Carbonic Anhydrase B†

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ABSTRACT: Human carbonic anhydrase B (HCAB), prepared by a new affinity chromatography procedure, was carboxymethylated exclusively at N^{τ} of its active-site histidine-200 using 90% [1-^{13}C]bromoacetate. The ^{13}C nuclear magnetic resonance signal of the covalently attached carboxylate was easily detected over the natural abundance background due to the other carbonyl and carboxyl carbons of this 29 000 molecular weight zinc metalloenzyme. Its chemical shift proved very sensitive to the presence of inhibitors in the active site and to variations in pH. Two perturbing groups with pK_a values of 6.0 and 9.2 were assigned to the modified histidine-200 itself and the zinc-bound water ligand, respectively, making use of ^{13}C NMR titration data on N^{τ}- and N^{π}-carboxymethyl-L-histidine model compounds. The results rule out histidine-200 as the critical group whose ionization controls the catalytic activity. They also strongly suggest an interaction of the carboxylate of the carboxymethyl group with either the zinc or its water ligand around pH 8, possibly explaining the basis for the major differences between HCAB and CmHCAB.

The structure and function of erythrocyte carbonic anhydrase (carbonate hydrolyase EC 4.2.1.1) has been extensively studied in recent years (Lindskog et al., 1971; Coleman, 1971; Lindskog and Coleman, 1973), but a number of outstanding mechanistic problems remain to be elucidated. Foremost among these is the identification of the essential group(s) responsible for the pH dependence of the catalytic activity (Khalifah, 1971; Khalifah and Edsall, 1972; Lindskog and Coleman, 1973; Pesando, 1975b; Martin, 1974). In addition, the roles of various active-site groups in binding the substrates and products remain obscure (Khalifah, 1971; Edsall and Khalifah, 1972). This situation persists despite the recent availability of high-resolution crystal structures for the two human isozymes HCAB[1] and HCAC (Liljas et al., 1972; Kannan et al., 1975; Notstrand et al., 1975). Carbonic anhydrase is also a zinc metalloenzyme, Co^{2+} being the only metal that can replace the essential zinc with full retention of catalytic activity (Lindskog et al., 1971; Coleman, 1971).

A number of ^{1}H nuclear magnetic resonance (NMR) studies have appeared in recent years on carbonic anhydrase (King and Roberts, 1971; Cohen et al., 1972; Pesando, 1975a,b; Gupta and Pesando, 1975; Campbell et al., 1974, 1975). The abundance of histidines in the active site and the apparent dependence of the catalytic activity on a group with $pK_a \sim 7$ makes such studies especially appealing, even though there are compelling reasons for considering the zinc-bound water ligand as the essential ionizing group (Lindskog and Coleman, 1973). The most detailed and informative study is that of Campbell and co-workers at 270 MHz in which tentative assignments were advanced for resolved signals from the 10–11 histidines of HCAB and HCAC. We have attempted a new and complementary approach to studying the active-site environment using ^{13}C nuclear magnetic resonance. We report here a study of the active-site ionizations of human carbonic anhydrase B exclusively carboxymethylated at N^{τ} of histidine-200 using 90% [1-^{13}C]bromoacetate. The enriched carboxyl gives rise to an easily observable ^{13}C NMR signal in CmHCAB that has proved to be a sensitive "probe" of the active site. These studies were greatly facilitated by the ability to rapidly prepare gram quantities of pure isozyme B from erythrocytes, so that we also report our adaptation of affinity chromatography for that purpose. A preliminary account of parts of this work has appeared elsewhere (Strader and Khalifah, 1976).

Experimental Procedure

Chemicals. Ninety percent [1-^{13}C]bromoacetic acid was purchased from Koch Isotopes. Its isotopic enrichment was confirmed by our mass spectral analyses. N^{τ}- and N^{π}-carboxymethyl-L-histidines were from Calbiochem. Bio-Rex 70 and CM Bio-Gel A ion exchangers and EDAC were obtained from Bio-Rad Laboratories. *p*-Aminomethylbenzenesulfonamide, *p*-carboxybenzenesulfonamide, and sodium cyanate were purchased from Aldrich Chemical Co. and used without further purification. DEAE-Sephadex A-50 was from Pharmacia. Trizma grade Tris base, Bistris, sodium barbital, imidazole, acetazolamide, and sulfanilamide were from Sigma. Enzyme grade ammonium sulfate was from Nutritional Biochemicals. CL 11 366 and ethoxzolamide (6-ethoxy-2-benzothiazolesulfonamide) were generous gifts from Dr. T. Maren, University of Florida. All other chemicals were analytical reagent grade whenever possible. Dialysis tubing was from A. H. Thomas.

Enzymes. Human carbonic anhydrase was prepared from freshly outdated erythrocytes generously donated by the Blood Bank of the University of Virginia Hospital. Erythrocytes were washed twice with cold saline and then lysed overnight by the

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[1] Abbreviations used are: HCAB or HCAC, human carbonic anhydrase isozyme B or C; CmHCAB, N^{τ}-carboxymethylhistidine-200 carbonic anhydrase B (human); τ- or π-CmHis, N^{τ}- or N^{π}-carboxymethyl-L-histidine (see also Figure 4 below; this is according to the recommended notation of the IUPAC-IUB CBN (1975)); EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Tris, tris(hydroxymethyl)aminomethane; Bistris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl) methane; CL 11 366, 5-benzenesulfonamido-1,3,4-thiadiazole-2-sulfonamide; DEAE, diethylaminoethyl.

addition of an equal volume of water and 5% toluene. After centrifugation, the hemolysate was extensively dialyzed against deionized water and then adjusted to pH 8.7–9.0 by the addition of solid Tris. Initially, we used DEAE-Sephadex chromatography to first remove the hemoglobin and then, on a second column, to separate the major isozymes by the method of Armstrong et al. (1966). The majority of our work, however, was on HCAB purified from hemolysate by the single-column, single-pass affinity chromatography procedure described under Results. Enzyme purity from the affinity chromatography preparations was established by DEAE-Sephadex or Bio-Rex 70 ion-exchange chromatography when necessary and by specific-activity measurements. Enzyme fractions from chromatography were concentrated by either vacuum dialysis or by precipitation through dialysis against an ammonium sulfate solution maintained at saturation during the dialysis. Enzyme concentrations were determined by the absorbance at 280 nm using known extinction coefficients (Lindskog et al., 1971).

Enzyme Assays. Enzyme activity and specific activity were followed by the Wilbur–Anderson colorimetric assay for CO_2 hydration (Rickli et al., 1964; Armstrong et al., 1966). This involves measuring the time it takes, following injection of CO_2 to a buffered enzyme solution, for the pH to drop by a certain amount measured by the color change of an indicator. The activity is then proportional to $(T_b/T_c) - 1$, where T_b is the uncatalyzed reaction time and T_c is the time in the presence of a given aliquot of enzyme.

Preparation of CmHCAB. HCAB has been discovered to react rapidly with either iodoacetate (Bradbury, 1969a) or bromoacetate (Whitney et al., 1967) to N^{τ}-carboxymethylate an active-site histidine residue. Peptide isolation work established that with either reagent histidine-200 in the amino acid sequence of Andersson et al. (1972) was exclusively modified when the reaction is carried out with low reagent concentration at neutral pH and for relatively short reaction times (Bradbury, 1969b; Andersson et al., 1969). We prepared ^{13}C-enriched CmHCAB by reacting HCAB with 90% [1-^{13}C]bromoacetate at 10–20 mM bromoacetate, pH 6.8–7.0, and for reaction times of only 3–8 h. The reaction was followed by monitoring the concomitant decrease in activity and it was observed to follow precisely the previously observed kinetics (Whitney et al., 1967). Reaction was quenched by adding excess 2-mercaptoethanol followed by extensive dialysis against many changes of cold buffer. In cases where reaction was terminated before 98% completion, the modified enzyme was chromatographed on Bio-Rex 70 (Bradbury, 1969a) to remove unreacted enzyme.

Preparation of Affinity Chromatography Gel. The affinity matrix was prepared by coupling the sulfonamide inhibitor *p*-aminomethylbenzenesulfonamide to the carboxyl-containing agarose ion-exchanger CM Bio-Gel A using the soluble carbodiimide EDAC (Hoare and Koshland, 1967). The procedure was essentially as described by Osborne and Tashian (1975) who coupled this inhibitor to CM Sephadex C-50. In a typical procedure, 3 g of the inhibitor dissolved in 50% aqueous acetone (100 mL) was added to 250 mL of swollen CM Bio-Gel A that had been washed with 50% acetone. The pH was adjusted to 4.80 with HCl and EDAC (5 g in 10 mL) was added dropwise with continuous stirring. The pH was maintained at 4.80 for 20 h, although the reaction pH stabilized after about 4 h. The gel was then exhaustively washed as described by Osborne and Tashian (1975) and equilibrated with the appropriate buffer for use.

Preparation of NMR Samples. Enriched CmHCAB was concentrated by vacuum dialysis down to 30–200 mg/mL (1–6 mM). Aliquots of 0.6–1.0 mL were then individually dialyzed against 250 mL of appropriate buffers, containing inhibitors if needed, usually overnight at room temperature. Buffers were 0.05–0.10 M Tris or Bistris-sulfate, titrated from the free base with H_2SO_4, and containing sufficient Na_2SO_4 or K_2SO_4 to bring the ionic strength to 0.20. The contents of the bags were then opened before use and centrifuged when necessary. They were augmented with two drops of D_2O to provide a lock signal and 2 μL of neat dioxane for internal chemical shift referencing. The pH of each sample was read just before and just after each ^{13}C NMR spectrum was taken, rarely drifting by more than 0.02 pH unit. Contact time with the electrode was minimized to avoid unnecessary KCl contamination (Campbell et al., 1974), although it should be noted that Cl^- inhibition of CmHCAB is some 30 times weaker than in HCAB (Whitney, 1973). Direct addition of acid or base to enzyme samples was strictly avoided due to their sensitivity to denaturation by such treatment. Enzyme concentration was also determined following each spectrum, as was the specific activity of noninhibited samples.

NMR Measurements. ^{13}C NMR spectra were taken on a JEOL PFT-100/EC 100 Fourier transform spectrometer equipped with a disk system and operating at 25.15 MHz for ^{13}C. Probe temperature was maintained at 25 °C for all runs and 10-mm flat-bottom sample tubes with Teflon vortex plugs were used. Chemical shifts are reported as ppm from Me_4Si, internal dioxane being assumed to be 67.40 ppm downfield from Me_4Si (Levy and Cargioli, 1972). Recycle times were 0.8 or 1.6 s, the flip angle being chosen to optimize a signal with a T_1 of 1.8–2.0 s. For the most concentrated samples (6 mM), a few minutes of accumulation was sufficient to obtain usable signal to noise ratios, while 1–2 h were needed for the least-concentrated samples. All spectra were fully ^1H noise decoupled.

Other Analytical Procedures. Absorption spectra were taken on a GCA/McPherson EU-707D double-beam spectrophotometer. An Orion 701 pH meter was used in conjunction with either Corning 476050 or Radiometer GK 2312C and GK 2322C combination glass electrodes. Mass spectra were run on a Hitachi Perkin-Elmer RMU-6E spectrometer.

Results

Affinity Chromatography. Several affinity chromatography matrices have been recently reported for the purification of carbonic anhydrase (Falkbring et al., 1972; Whitney, 1974; Wistrand et al., 1975; Osborne and Tashian, 1975). The method of Osborne and Tashian (1975) appears to produce gels with the highest capacity for the enzyme, but it utilizes a matrix of CM Sephadex C-50 that has a bed volume that is quite sensitive to wide variations in pH and ionic strength. We consequently explored the use of CM Bio-Gel A that has excellent stability to such variations that are frequently encountered in affinity chromatography. The affinity gel we have prepared has completely met our expectations in this regard and has shown a binding capacity of 15–20 mg of carbonic anhydrase/mL of bed volume, similar to the values reported by Osborne and Tashian (1975). However, the excellent flow rates normally found with it drop significantly during the passage of hemolysate. Batch adsorption of the enzyme from hemolysate was then attempted with the results shown in Figure 1A. Addition of gel to hemolysate with continuous stirring requires about 1 h for complete adsorption, representing a great savings in time when processing several liters of hemolysate. Gel is subsequently separated from hemolysate

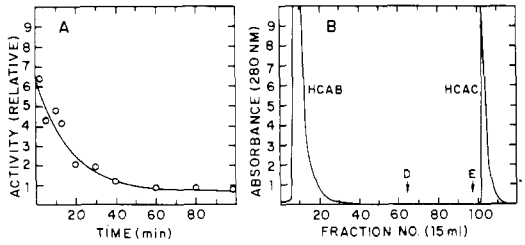

FIGURE 1: Affinity chromatography of human carbonic anhydrase: (A) kinetics of batchwise (stirred) adsorption of carbonic anhydrase from hemolysate; (B) elution of isozymes following washing of affinity gel to remove hemoglobin, HCAB being eluted with KI beginning at fraction 0 and HCAC being eluted with NaN_3 starting with the fraction indicated by E (see text for details).

by filtration on a Buchner funnel and is washed with 0.75 M ionic strength Tris-sulfate of pH 9 until visibly free from hemoglobin. It is then poured into a column and washed with 0.75 M ionic strength Tris-sulfate buffer at pH 7.50 until the absorbance of 280 nm is negligible. Elution of the major isozyme B (+A) of low specific activity is achieved selectively (Figure 1B) by including 0.2 M KI inhibitor in the buffer. High specific activity HCAC is then selectively eluted with 0.4 M NaN_3 (caution in disposal) in Bistris-sulfate buffer of pH 7.0 after a prior wash with high ionic strength Bistris-sulfate of pH 7.0 not containing the powerful azide inhibitor. The whole procedure beginning with hemolysate adsorption can be done in 1 working day.

The purity of the isozymes was further checked by DEAE-Sephadex and Bio-Rex 70 ion-exchange rechromatography. In agreement with Wistrand et al. (1975) and Osborne and Tashian (1975), we found the HCAC to contain a trace (~0.2%) of similar high specific activity material eluting close to the position of HCAB on DEAE-Sephadex, but no HCAB. On the other hand, the HCAB fraction contained a little less than 10% of the A isozyme, as expected. The latter was not routinely removed in our work, since it is presumed to be identical to HCAB except for the probable loss of a nonessential amide group (Lindskog et al., 1971) and since its removal did not detectably affect any of our results.

^{13}C NMR Spectrum of Enriched CmHCAB. It was important to first ascertain whether the ^{13}C NMR signal from the *single carbon* of the enriched carboxylate of CmHCAB could be readily detected and identified. Figure 2 shows a 200 ppm ^{13}C NMR spectrum of enriched CmHCAB. The low-field carbonyl and carboxyl carbon region near 175 ppm is seen to have a sharp, dominant resonance that must emanate from the covalently attached carboxylate, since it is absent in HCAB or in CmHCAB prepared with nonenriched bromoacetate.[2] The inset shows more clearly that the enriched carboxylate resonance is about three times larger than the envelope of the natural abundance signals of the carbonyls and carboxyls of the enzyme. The chemical shift of the enriched carbon lies close to the position of the corresponding carboxyl of carboxymethylated histidine, as discussed in detail below. The signal also shows no splittings, nor is there any indication of minor peaks. This is true under the variety of conditions (pH, inhibition) discussed below where the signal is substantially shifted. It is also not due to unreacted reagent, since the spectrum is unaltered when CmHCAB is extensively chromatographed

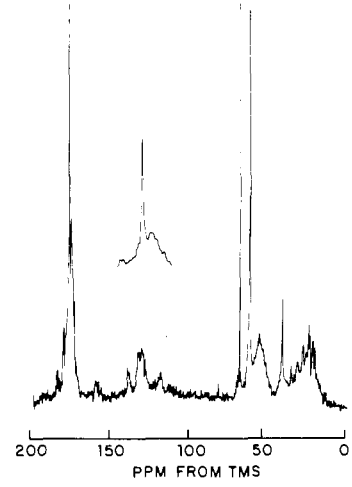

FIGURE 2: ^{13}C NMR spectrum of 5.6 mM CmHCAB prepared with 90% [1-^{13}C]bromoacetate. The sharp peaks at 61.0 and 67.4 ppm are due to the Tris buffer and dioxane, respectively. The sharp peak (off scale) at low field (174.5 ppm) in the carbonyl region is from the single enriched carboxyl covalently attached to the enzyme. The inset shows the carboxyl region on scale (but slightly expanded) to show relation of the single enriched carboxyl signal to the envelope of the natural abundance signals of the carbonyl carbons of the enzyme. The pH was 7.65 and the spectrum represents 23 000 transients at 1.7-s recycle and 65° flip angle.

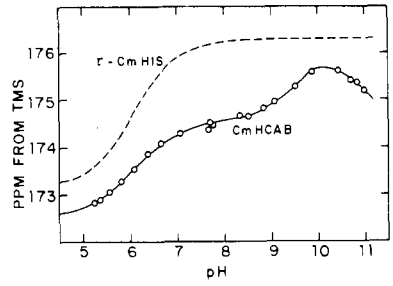

FIGURE 3: ^{13}C NMR titration of the enriched carboxylate of CmHCAB at 25 °C and 0.2 ionic strength. Buffers were Bistris or Tris-sulfate. Shifts are given as downfield from tetramethylsilane.

on various ion-exchange columns. We conclude that we are observing the signal from a covalently bound enriched carboxylate as a result of the exclusive carboxymethylation at N^{τ} of histidine-200 of the amino acid sequence of Andersson et al. (1972). This situation is quite different from the nonspecific alkylations carried out with [2-^{13}C]bromoacetate on myoglobin (Nigen et al., 1973) and cytochrome *c* (Eakin et al., 1975), where much higher reagent concentrations (0.2 M) and longer reaction times (6–8 days) were employed, resulting in a wide variety of alkylated products and ^{13}C NMR signals.

Titration Shifts of Enriched CmHCAB. The modified histidine-200 is part of the active site of HCAB (Kannan et al., 1975) and it was of interest to see whether the enriched carboxylate signal was affected by active-site ionizations. Figure 3 shows that the chemical shift is quite sensitive to pH when CmHCAB is titrated between pH 5.2–11.0. Three separate inflections are detected. The behavior between pH 5.2 and 10.5 shows upfield protonation shifts, while above pH 10.5 an upfield shift on deprotonation is observed. It was not possible to extend the data above pH 11.0, since the enzyme was found to gel and lose activity during the spectral measurements. In all the experiments between pH 5.2 and 11.0, the enzyme was fully active after spectra were run.

The pH dependence of the chemical shift below pH 10.5

[2] The ^{13}C NMR spectra of HCAB and unenriched CmHCAB are identical, as would be expected. Although not shown here, a typical ^{13}C NMR spectrum of HCAB can be found in Feeny et al. (1973).

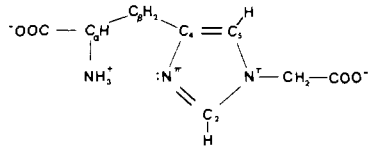

FIGURE 4: Structure and nomenclature for N^{τ}-carboxymethyl-L-histidine.

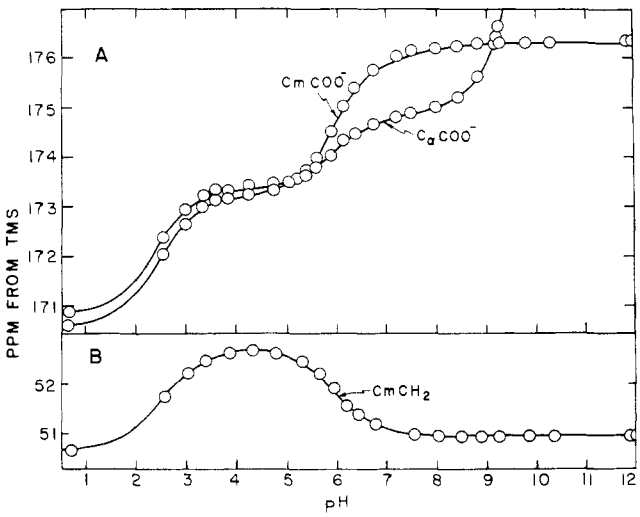

FIGURE 5: ^{13}C NMR titration shifts of τ-CmHis: (A) carboxyl carbons; (B) methylene carbon of the carboxymethyl group. Shifts are given as downfield from tetramethylsilane.

could be quantitatively fit to a model in which the shift is perturbed by two independent ionizations:

$$EH_2^{2+} \rightleftharpoons EH^+ + H^+ \qquad pK_2$$

$$EH^+ \rightleftharpoons E + H^+ \qquad pK_1$$

Here E is the enzyme form at pH 10.5 and EH_2^{2+} is that predominating at pH 5. The fraction of each form at any pH is given by the well-known pH functions:

$$f^{\circ} = [E]/[E]_{total} = (1 + [H^+]/K_1 + [H^+]^2/K_1K_2)^{-1}$$

$$f^{+} = [EH^+]/[E]_{total} = (1 + K_1/[H^+] + [H^+]/K_2)^{-1}$$

$$f^{2+} = [EH_2^{2+}]/[E]_{total} = (1 + K_2/[H^+] + K_1K_2/[H^+]^2)^{-1}$$

Assuming rapid exchange on the NMR time scale among the species, the observed chemical shift will be given by:

$$\delta_{obsd} = f^{\circ}\delta^{\circ} + f^{+}\delta^{+} + f^{2+}\delta^{2+}$$

where δ°, δ^{+}, and δ^{2+} are the chemical shifts of the enriched carboxylate in E, EH^+ and EH_2^{2+}, respectively. The data are fit to a ±0.03-ppm standard error making the choice of $pK_1 = 9.02$, $pK_2 = 6.00$, $\delta^{\circ} = 175.70$ ppm, $\delta^{+} = 174.45$ ppm, and $\delta^{2+} = 172.55$ ppm. The pK_1 ionization is thus accompanied by an upfield protonation shift of 1.90 ppm, while the pK_2 ionization causes an upfield protonation shift of 1.25 ppm. No attempt was made to fit the data above pH 10.5, since the insufficient pH range prevented the completion of that process and the determination of whether it represented a single ionization.

Titration Shifts of Carboxymethyl-L-histidines. A ^{13}C NMR titration was carried out on both N^{τ}-carboxymethyl-L-histidine (τ-CmHis) and N^{π}-carboxymethyl-L-histidine (π-CmHis). The structure of the former is shown in Figure 4,

TABLE I: ^{13}C Imidazole Protonation Shifts[a] of *N*-Alkyl-L-histidines.[b]

Compd	Carbon Position			
	C-4	C-5	C*mCOO⁻	CmC*OO⁻
τ-CmHis	−6.7	+2.1	+1.8	−3.1
τ-MeHis[c]	−6.8	+2.1		
π-CmHis	+2.3	−8.0	+1.5	−3.2
π-MeHis[c]	+2.3	−(7.1)[c]		
CmHCAB (p*K* 6.0)				−1.9[d]
CmHCAB (p*K* 9.2)				−1.2[d]

[a] Downfield protonation shifts are positive, in parts per million. [b] See Figure 4 and footnote 1 of text for numbering and nomenclature. Asterisk denotes carbon (methylene or carboxyl) of carboxymethyl group being referred to. [c] Data of Reynolds et al. (1973). It is obvious by perusal of the discussion and Figure 2 of this reference that all the entries in their Table II for 1-MeHis and 3-MeHis (π-MeHis and τ-MeHis, respectively, by our nomenclature) have been inadvertently interchanged. In addition, the −7.1 ppm entry for C-5 of (corrected) π-MeHis does not agree with their Figure 2. The latter suggests a value closer to −8.0, greatly improving the agreement with our value for π-CmHis. The apparently erroneous value has been quoted in the literature (Deslauriers et al., 1974). [d] These shifts correspond to the inflection amplitudes of the titration data in Figure 3 of the text. Only the pK_a 6.0 inflection has been attributed to imidazole ionization (see Discussion).

and it can be seen that there are two carboxyls that should have similar intensities in this nonenriched model compound. Figure 5A shows their ^{13}C NMR titration behavior. There are two crossovers in the titration, but the signal from the carboxymethyl carboxyl can be easily assigned, since it is found, as expected, to be insensitive to the ionization of the α-amino group above pH 8. This carboxyl, which is structurally analogous to the enriched carbon of CmHCAB, undergoes upfield shifts during the protonation of the imidazole ring (pK_a ~6.1) and during its own carboxyl protonation below pH 4. The imidazole protonation shift of this carboxyl is also shown as the dashed curve in Figure 3, where it is seen to cover the same chemical-shift range as the titrating signal from CmHCAB. However, it is obvious that the titration shift of the model compound carboxyl upon imidazole protonation is larger than any of the individual inflections seen with CmHCAB. These titration shifts are given in Table I which also includes the corresponding data of the carboxymethyl carboxyl (CmC*OO⁻) of π-CmHis.

There are other aspects of interest in the titration data of these model compounds. The approximate[3] pK_a values that we found for the ring ionization of τ-CmHis (~6.1) and π-CmHis (~6.5) are not too different from the pK_a of histidine itself (6.1-6.2) at an ionic strength of 0.2 M (Sachs et al., 1971). Thus, carboxymethylation by itself should not lead to appreciable changes in the pK_a of the ring. A similar situation also occurs when histidine is internally situated in a tripeptide (Whitney et al., 1967). The ^{13}C NMR titration shifts of the C-4 and C-5 carbons of these model compounds (Table I) are relevant to the proposal of Reynolds et al. (1973) for utilizing the shifts of such carbons to determine the ring tautomeric equilibrium position in *unsubstituted* histidines. Alkylation at N^{τ} or N^{π} produces structurally "frozen" tautomers analogous to τ-H or π-H tautomers of histidines. While Reynolds

[3] The pK_a values should not be considered as accurate, since no effort was made to keep the ionic strength or concentration constant during the titrations.

et al. (1973) used the τ- and π-methylhistidine pair, the carboxymethylhistidines provide a second suitable such pair. The C-4 and C-5 ^{13}C NMR titration shifts (Table I) show the generally excellent quantitative agreement between the two pairs of compounds, thus strengthening the validity of the proposed tautomer shifts (see, however, footnote c to Table I). Finally, the titration shifts of the methylene carbon of the carboxymethyl group of the model compounds are also of interest, since [2-^{13}C]bromoacetate is commercially available and has, in fact, been used in previous carboxymethylation studies of proteins (Nigen et al., 1973; Eakin et al., 1975). Figure 5B shows that the ^{13}C NMR titration of this carbon in τ-CmHis behaves quite differently from the carboxyl carbon (Figure 5A) in that it undergoes a *downfield* shift upon imidazole protonation. In addition, its titration shift is 1.75 ppm (1.50 ppm in π-CmHis), which is just over one-half the value found for the carboxymethyl carboxyl. This makes it possibly a less sensitive probe than the carboxyl.

Inhibition Studies. The chemical shift of the enriched carboxylate has proven very sensitive to the presence of inhibitors in the active site. At pH 7.9, both downfield (positive) and upfield (negative) inhibitor shifts have been observed. Typical shifts are: nitrate (+0.83 ppm), azide (0.39), iodide (0.29), imidazole (0.22), cyanate (−0.44), *p*-aminomethylbenzenesulfonamide (−1.11), sulfanilamide (−1.58), ethoxzolamide (−1.84), acetazolamide (−2.57), CL 11 366 (−3.05), and *p*-carboxybenzenesulfonamide (−3.88). With the exception of imidazole, the above values were obtained at inhibitor concentrations at least eight times the approximate K_i (Whitney and Brandt, 1976; Whitney, 1970, 1973, 1974; Taylor et al., 1970; Taylor and Burgen, 1971). Imidazole is a unique competitive inhibitor of CO_2 in HCAB (Khalifah, 1971), and the above results obtained at 0.2 M imidazole suggest that it may also inhibit CmHACB. The inhibitor shifts can be due to a number of different factors, such as changes in the pK_a values of groups causing the inflections in Figure 2 (Khalifah, 1977), so that no interpretations are attempted here in the absence of complete titrations of CmHCAB–inhibitor complexes.

Discussion

We have successfully utilized the techniques of highly specific chemical modification to introduce an isotopically enriched ^{13}C group into the active site of HCAB for use as an NMR probe. This approach overcomes the sensitivity problems associated with observing single carbon signals of proteins and, more importantly, entirely circumvents resolution and assignment difficulties inherent to studies of such macromolecules (Komoroski et al., 1976). We have thus exclusively and selectively carboxymethylated the active-site histidine-200 with 90% [1-^{13}C]bromoacetate, making use of this previously studied reaction (Bradbury 1969a,b; Whitney et al., 1967; Andersson et al., 1969). The signal from the enriched carboxyl, now in the active site, has proved surprisingly easy to detect over the natural abundance background due to some 314 other similar carbonyl and carboxyl carbons in this 29 000 molecular weight enzyme (Figure 2). The ability of this ^{13}C NMR "probe" to monitor active-site events is perhaps most dramatically illustrated by the significant shifts it experiences in the presence of many known carbonic anhydrase inhibitors, whether of the sulfonamide, monoanion, or metal-poison type. These inhibitor shifts provide a strong basis for believing that the pH-dependent shifts of the free enzyme shown in Figure 3 must be due to ionizations intimately involved in the active site. It is pertinent to recall here that, although carboxymethylation significantly reduces the catalytic activity, the residual activity is by no means negligible, being between 2–20% depending on pH and substrate used. The residual *esterase* activity has been convincingly shown by Whitney (1970) to be an intrinsic property of CmHCAB, the greatest difference being the shift of the pK_a of the catalytically essential ionizing group from 7.3 in HCAB to 9.2 in CmHCAB. The residual CO_2 hydration activity shows more complex pH dependence but has been rationalized in terms of the same type of mechanism as that operative for HCAB (Khalifah and Edsall, 1972).

In light of the above, it is gratifying to find that the carboxylate of CmHCAB does indeed show the influence of an ionizing group of pK_a 9.2 (Figure 3). Presumably, this is the essential catalytic group. There have been a number of suggestions regarding its identity, these being usually either an active-site histidine (ligand or nonligand) or a metal-coordinated water ligand that ionizes to a hydroxyl (Lindskog et al., 1971; Pesando, 1975b; but see, however, Martin, 1974). The visible absorption spectrum of the active Co^{2+}-substituted carbonic anhydrases shows large pH-dependent changes that correlate excellently with the pH dependence of the catalytic activity and its inhibition (Lindskog, 1966). Furthermore, the pK_a of this spectral change increases from about 7.3 in HCAB to 9.2 in CmHCAB (Whitney, 1970; Taylor et al., 1970), in parallel with the above-mentioned shift of the apparent pK_a of the esterase activity of *either* Zn^{2+}- or Co^{2+}-CmHCAB (Whitney et al., 1967; Whitney, 1970). These and other observations (cf. Lindskog and Coleman, 1973) suggest that the essential ionizing group is a metal-bound water-hydroxyl ligand.

The upfield protonation shift of pK_a 9.2 experienced by the carboxylate is consistent with an increase in positive charge in its vicinity (Batchelor, 1975; Batchelor et al., 1975), as might occur if a zinc-bound hydroxyl in its vicinity became protonated. Presuming that the modified histidine-200 is deprotonated above pH 8 (see below), it is evident that the chemical shift at pH 8 appears more "abnormal" relative to τ-CmHis than at pH 10 (see Figure 3). This suggests that the carboxylate is oriented towards the zinc at pH 8 but perhaps not at pH 10. Since the visible spectral studies of Co^{2+}-CmHCAB (Whitney, 1970; Taylor et al., 1970) seem to rule out direct inner-sphere coordination of the carboxymethyl carboxyl at either low or high pH, the presumed interaction may have to occur through the intervening water ligand. This suggested interaction may thus provide the molecular basis of the higher pK_a of the zinc water in CmHCAB as compared to HCAB. The enzyme derivative produced by alkylating histidine-200 with iodoacetamide also has a higher pK_a (8.2) in its activity–pH profile or its Co^{2+} spectral change (Whitney, 1973), so that the amide group could be similarly interacting with the metal or its water ligand. The higher pK_a in the case of CmHCAB may be due to a stronger interaction of the zinc with the negatively charged carboxylate. In view of these possibilities, a ^{13}C NMR titration of metal-free CmHCAB should prove quite interesting, and it is currently being attempted in this laboratory.

The group with pK_a 6.0 affecting the carboxylate chemical shift is almost certainly histidine-200 itself that can now be protonated at N^{π} only. Figure 3 indicates that an upfield protonation shift of similar magnitude is to be expected in the absence of dominating environmental effects that could arise in the enzyme active site. The position of the inflection at pH 6.0 strongly implicates a histidine, and we have already pointed out above that carboxymethylation by itself should not greatly

alter the ring pK_a. The only data on the pK_a of histidine-200 come from the ^1H NMR study of *native* HCAB by Campbell and co-workers (1974). They deduced a pK_a of 6.14 for a titrating resonance that they tentatively assigned to the C-2 H of histidine-200. Unfortunately, they were unable to observe the signal of this residue in CmHCAB, making a direct comparison between the two studies not possible. In any case, we feel our assignment, based on the behavior of τ-CmHis, to be much more direct and certain than possible with ^1H NMR. Thus, carboxymethylation of histidine residues in conjuction with ^{13}C observations, as in this study, should yield an excellent route to determining the pK_a of carboxymethylated histidines. It may also suggest approximate pK_a values for the corresponding unmodified histidines, but this would be more tenuous.

The titration shift of the pK_a 6.0 inflection of the enriched carboxylate and the imidazole protonation shift of the corresponding carboxyl in the model compound do differ, however, by about 30%. Comparison of the absolute chemical shifts of the two (Figure 3) shows that the diminished protonation shift in the enzyme is probably due to the "abnormal" chemical shift at the pH 8 plateau, since the chemical shift near pH 5 appears more normal relative to the model compound. In terms of the possibility raised above of an interaction between the carboxylate and the zinc or its water ligand, one would conclude that the interaction is abolished when histidine-200 is protonated. This would occur if protonation of the histidine necessitated the rotation of the ring, forcing movement of the carboxylate and a disruption of its interaction with the metal. This coupling between the histidine ionization and the carboxylate-zinc interaction would be expected to lower the pK_a of histidine-200 in CmHCAB compared to HCAB. Since this is apparently not observed, the above explanation may be incorrect, or, alternatively, compensating effects also exist to affect the pK_a of histidine-200.

The high pH (10.5–11.0) upfield shift seen in Figure 3 is rather difficult to assign, especially since its direction is unusual. The enzyme loses activity and forms gels above pH 11, so that the upfield shift above pH 10.5 could represent the beginning of that process. Time-dependent losses in activity above pH 11 have been previously noted in spectrophotometric and potentiometric titrations of HCAB (Riddiford, 1964; Riddiford et al., 1965), concomitant with unmasking of tyrosines. However, the activity of CmHCAB is retained up to pH 11, so that the observed shift could also be due to the influence of a simple, noncooperative ionization with a $pK_a \gtrsim 10.7$. It may be relevant here that a perturbing group with pK_a of 10.8 has been observed by Whitney (1973) to influence the visible absorption spectrum of Co^{2+}-substituted HCAB alkylated with iodoacetamide at histidine-200. Qualitatively similar but much weaker effects are discernible in the corresponding data on CmHCAB (Whitney, 1970), although in the latter case they may be within the experimental error. Further experiments are obviously required to clarify these high-pH changes.

In conclusion, we have demonstrated that histidine-200 is unlikely to be the essential group whose ionization controls the activity of CmHCAB and, by inference, HCAB. Our ^{13}C NMR approach can also be extended to determining the microscopic pK_a values of active-site groups in *complexes* of the enzyme with inhibitors and certain substrates. In fact, such studies have already led to the identification of histidine-200 as the group with pK_a 6 recently reported by Whitney and Brandt (1976) to influence inhibitor binding in CmHCAB (Khalifah, 1977). Finally, it should be noted that the choice of enriching a carboxyl carbon was instrumental in leading to its facile detection over the natural abundance ^{13}C NMR signals. Carbons lacking *directly* bonded protons have inefficient dipolar relaxation and have much narrower resonances than protonated carbons (Doddrell et al., 1972; Oldfield et al., 1975). These considerations are very important in contemplating similar studies on even larger macromolecules (cf. Browne et al., 1976).

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