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Carbon-13 Nuclear Magnetic Resonance as a Probe of Side Chain Orientation and Mobility in Carboxymethylated Human Carbonic Anhydrase B†

Antonius J. M. Schoot Uiterkamp, Ian M. Armitage,* James H. Prestegard, John Slomski, and Joseph E. Coleman*

ABSTRACT: ^{13}C NMR spectra of [1-^{13}C]- and [2-^{13}C]carboxymethyl His-200 human carbonic anhydrase B have been obtained as a function of pH and in the presence and absence of the active site Zn(II) or Cd(II) ion. Chemical shifts of the 1-^{13}C show that the carboxyl is sensitive to two ionization processes, with apparent pK_as of 7.2 and 9.9, respectively. These are assigned to the deprotonation of the N^{π} of His-200 and the breaking of the coordination bond between the carboxyl oxygen and the Zn(II) ion, respectively. Assignment of the lower pK_a to that of the N^{π} is supported by the observation of this same ionization in the chemical shift of the 2-^{13}C resonance showing the signal from the methylene carbon to undergo the same upfield shift as is observed on the ionization of the N^{π} in N^{τ}-carboxymethylated histidine. The high pH ionization process is not reflected in the resonance of the methylene carbon. No changes in the chemical shifts vs. pH are observed for both the [1-^{13}C]- and [2-^{13}C]carboxymethyl apocarbonic anhydrase, suggesting that the pK_a of the N^{π} has shifted at least 1 pH unit to acid pH, and must reflect significant conformational changes in the active center. Cd(II) carboxymethyl carbonic anhydrase shows ^{13}C chemical shifts identical with those of the apoenzyme. Since the Cd(II) at the active site is known to bond external donor groups very weakly, the data suggest that the changes in conformation are related to Zn(II)-carboxylate coordination. Changes in the mobility of the carboxymethyl group have been assessed by a relaxation analysis which relates the relative line widths of the central and outer lines of the [^{13}C]methylene triplet to the internal rotational motion of the group relative to the protein. At neutral pH this group shows no internal motion, compatible with its coordination to the active site metal ion. At pH 10.6 significant internal motion is present, compatible with breaking of the coordination bond in competition with ^{-}OH binding.

Human erythrocytes contain two forms of carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1), a low and a high activity isozyme, designated carbonic anhydrase B and carbonic anhydrase C, respectively (Lindskog et al., 1971). These zinc metalloenzymes have a mol wt of ~30 000 and crystal structures at a resolution of 2.2 Å for B and 2.0 Å for C are known (Liljas et al., 1972; Kannan et al., 1975). Carbonic anhydrase catalyzes the reversible hydration of CO_2 as well as a number of other hydrolysis and hydration reactions involving carbonyl groups, including the hydrolysis of several esters (Pocker & Watamori, 1971).

All isozyme and species variants studied thus far show a pH-rate profile sigmoid to high pH with midpoints from pH 6.9 to 7.5 depending on isozyme and conditions, e.g., the presence of anions (Lindskog, 1966; Coleman, 1967a). To a first approximation the pH-rate profile is represented by a single ionization. The ionization resulting in the active form of the enzyme is thought to be that of either a Zn(II)-coordinated water molecule or an amino acid side chain adjacent to the metal ion (see Lindskog & Coleman, 1973, for discussion).

The B isozyme is much more sensitive to inhibition by anions than the C isozyme (Maren et al., 1976). The structure of the B isozyme also differs from that of the C isozyme in that the active site cleft of the B isozyme contains a histidine residue at position 200 which can be selectively carboxymethylated at the N^{τ} position by iodo- or bromoacetate. Carboxymethylation of His-200 results in a shift of the pK_a of the activity-linked group to pH ~9.0. Maximum activity of the carboxymethylated enzyme is ~20% that of the native enzyme. The carboxymethylation of the enzyme requires that iodo- or bromoacetate first be bound in reversible fashion to the active center, a reaction that depends on the presence of the metal ion (Whitney et al., 1967; Bradbury, 1969). These observations were combined in a specific model for the carboxymethylation reaction by Coleman (1971) in which the carboxyl of the reagent coordinates the metal ion, which leaves the N^{τ} of His-200 in a position for nucleophilic attack at the iodinated carbon. After reaction the carboxymethyl group remains coordinated to the Zn(II) ion at neutral pH, while ^{-}OH competes successfully at high pH regenerating the active enzyme and accounting for the shift in the apparent pK_a of the activity-linked group to pH ~9. Powerful anionic inhibitors like CN^{-} and sulfonamide compete with the carboxyl for the coordination site at neutral pH for which there is spectral evidence using the d-d transitions of the Co(II) enzyme (Coleman, 1971, 1975). In order to further test the validity of this hypothesis we have carried out a ^{13}C NMR study of carboxymethylated human carbonic anhydrase B (Cm HCAB[1]), using iodoacetate enriched in either the carboxyl carbon (Cm[1-^{13}C]HCAB) or the methylene carbon (Cm[2-^{13}C]HCAB).

The metal ion dependence of the chemical shifts of these carbons as functions of pH support an interaction of the car-

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[1] Abbreviations used: HCAB, human carbonic anhydrase B; CmHCAB, carboxymethyl human carbonic anhydrase B.

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boxyl with the metal ion. Protonation of the N^{π} of carboxymethyl His-200 is also shown to require the zinc ion, suggesting that the conformation of His-200 in the carboxymethyl form is altered significantly by metal ion binding. The resonance triplet from the [^{13}C]methylene has been used to apply a new method for characterizing anisotropic motion of the carboxymethyl group as a function of pH and metal ion by analysis of the transverse relaxation in this AX_2 nuclear spin system. These data also suggest reversible coordination of the carboxyl group to the metal ion. ^{13}C NMR of the zinc-containing [1-^{13}C]carboxymethyl enzyme has also been reported by Khalifah and co-workers (Khalifah, 1977; Khalifah et al., 1977).

Materials and Methods

Human carbonic anhydrase B was isolated according to Armstrong et al. (1966). Specific carboxymethylation of His-200 was carried out according to Bradbury (1969). Best results were obtained with protein concentrations of about 0.2 mM, a label to protein molar ratio of 40:1 with an incubation period of 48 h, 4 °C, in the dark. Zinc was removed from the carboxymethylated enzyme with pyridine-2,6-dicarboxylic acid by a modification of the method of Hunt et al. (1977). The 0.1 M sodium phosphate buffer used in their method invariably resulted in protein denaturation and was therefore substituted with 10 mM sodium phosphate. Up to 40 mL of 0.2 mM labeled human carbonic anhydrase B was dialyzed under stirring at 20 °C vs. two successive 1 L volumes of 50 mM pyridine-2,6-dicarboxylic acid in 10 mM sodium phosphate, pH 7. Zinc removal was monitored with atomic absorption and was complete, <3% remaining, in 1 day. Apoenzyme samples were extensively dialyzed vs. the appropriate buffer to remove the chelator.

In reconstitution experiments with $ZnCl_2$ or $CdCl_2$, metal addition was carried out in 10 mM Tris–sulfate, pH 7. Protein samples were contained in a sodium sulfate solution of 0.1 ionic strength containing 10 mM sodium phosphate when indicated. Titrations were carried out either by dialysis against buffer solutions at different pH values or by repeated additions of 2-μL aliquots of 0.1 N NaOH, which was freed of metals by treatment with Chelex 100. Metal-free glassware and buffers were used throughout (Harris & Coleman, 1968). pH was measured with a 3-mm combination electrode with a K_2SO_4 salt bridge (Wilmad) and pH values are reported as actual meter readings. Pyridine-2,6-dicarboxylic acid was from Sigma and 90% [1-^{13}C]- and 90% [2-^{13}C]iodoacetate were purchased from Koch. The ^{13}C enrichment and chemical purity of the labeled iodoacetates were confirmed by mass spectrometry employing the methyl esters. ^{113}CdO, 95 atom % excess, was purchased from Oak Ridge National Laboratories. ^{113}CdO was dissolved in concentrated HCl and taken to dryness before dilution in the appropriate buffers; hence it was added with a small amount of chloride. Circular dichroism in the aromatic absorption bands of the carboxymethylated protein was determined between 300 and 260 nm on a Cary 61 spectropolarimeter. Protein concentrations were determined from measurements of the optical densities at 280 nm using a molar absorptivity of 4.90×10^{-4} M^{-1} cm^{-1} (Armstrong et al., 1966).

^{13}C NMR of the labeled enzymes as a function of pH was done with a FT Bruker HFX 90-MHz spectrometer at 22.6 MHz. Broadband proton noise decoupling (10 W) was employed. Data acquisition employed a spectral width of 5000 Hz with 2048 accumulated data points and a pulse repetition rate of 0.5 s.

^{1}H and ^{13}C (transverse relaxation study) FT NMR spectra were obtained on a Bruker HX 270 at 270 and 67 MHz, respectively. D_2O, present as either bulk solvent or in a 3-mm coaxial capillary insert, provided the deuterium lock for field-frequency stabilization. ^{13}C measurements were made at 27 ± 2 °C on 1–1.5-mL samples contained in 10-mm flat-bottomed sample tubes. For ^{1}H measurements, 5-mm sample tubes were used. Protein concentrations in NMR experiments varied from 2 to 6 mM. ^{13}C chemical shifts are expressed relative to the resonance position of tetramethylsilane. Dioxane (67.4 ppm downfield from tetramethylsilane) contained in the D_2O capillary insert was used as an internal chemical shift reference in the titration studies. pK_a values were determined by a linear least-squares fit of the chemical shift data as a function of pH.

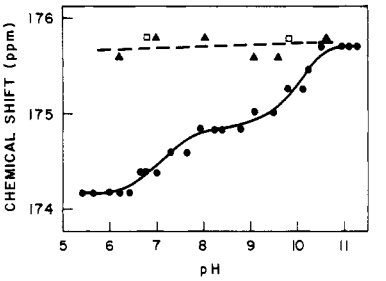

FIGURE 1: pH dependence of chemical shift of ^{13}C resonance of [1-^{13}C]carboxymethyl human carbonic anhydrase B. Conditions were: sodium sulfate solution of 0.1 ionic strength containing 10 mM sodium phosphate; (●) Zn(II) enzyme; (▲) apoenzyme; (□) Cd(II) enzyme.

Results

^{13}C NMR of [1-^{13}C] Carboxymethyl Human Carbonic Anhydrase B as Function of pH. The chemical shift values for the ^{13}C resonance of carboxymethyl human B enzyme enriched in the carboxyl group at representative pH values between pH 5.3 and 11.3 are plotted in Figure 1. The chemical shift values at the three observed plateau regions are 174.2, 174.8, and 175.7 ppm for pH 5.3, 8.5, and 11.3, respectively. The two pK_a values fitting this curve are 7.17 ± 0.08 and 9.91 ± 0.08. In contrast to the behavior of the zinc enzyme, no dependence of the chemical shift on pH is observed for the apocarboxymethyl enzyme. The chemical shift of the apo form remains 175.7 ppm down to pH 6, a chemical shift identical with that of the Zn(II) enzyme above pH 11 (Figure 1). To confirm whether the labeled apoenzyme could be reconstituted, $ZnCl_2$ was added to the apocarboxymethyl enzyme at pH 7.0. This results in a 1.3-ppm upfield shift in the resonance of the carboxyl carbon to the same chemical shift and line width as observed in "native" carboxymethyl human carbonic anhydrase B.

In contrast, the addition of Cd(II) to the apoenzyme at pH 6.8 gives rise to the same chemical shift and line width as observed in the apoenzyme (Figure 1). Raising the pH of this solution to 9.9 results in no change in chemical shift. The binding of Cd(II) to the carboxymethyl apoenzyme was confirmed by adding 1.5 equiv of Cd(II) to the apoenzyme and dialyzing 1 mL of the enzyme against two 1-L volumes of buffer for 24 h each. Atomic absorption spectroscopy showed 1 mol of Cd(II) per mol of enzyme to remain after dialysis. The preservation of the native structure in the apocarboxymethyl enzyme was monitored by determining the circular dichroism of the aromatic absorption bands which show an extremely complex ellipticity typical of the native enzyme.

To further explore molecular structure at the active site, the apocarboxymethyl enzyme was reconstituted with ^{113}Cd(II). This isotope of nuclear spin ½ could potentially couple to the [^{13}C]carboxyl carbon if they shared a common bonded oxygen

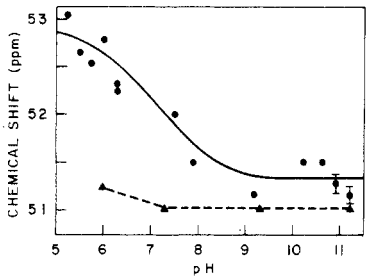

FIGURE 2: pH dependence of chemical shift of ^{13}C resonance of [2-^{13}C]carboxymethyl human carbonic anhydrase B. Conditions were: sodium sulfate solution of 0.1 ionic strength; (●) Zn(II) enzyme; (▲) apoenzyme.

atom. No evidence of coupling was observed (see Discussion).

^{13}C NMR of [2-^{13}C]Carboxymethyl Human Carbonic Anhydrase B as a Function of pH. The chemical shift values for the resonance of the [^{13}C]methylene carbon of the carboxymethyl His-200 between pH 5.3 and 11.5 are plotted in Figure 2. In contrast to the two transitions observed for the resonance of the carboxyl carbon, only the low pH transition is observed in the case of the methylene carbon. The pK_a, 6.8 ± 0.3, derived from the least-squares fit assuming a single ionization is similar to that of the first pK_a for the [^{13}C]carboxyl. The larger standard deviation for the methylene pK_a compared with the carboxyl pK_a is due to the increased uncertainty in defining the chemical shift for this resonance as a result of its increased line width and the large number of overlapping methylene resonances from the protein. Increasing pH results in an upfield shift of the resonance of the methylene labeled protein as opposed to the downfield shift observed in the carboxyl labeled protein. The magnitude and direction of the chemical shift are the same as reported by Khalifah et al. (1977) for the methylene carbon in the model compound, N^{τ}-carboxymethylhistidine. As observed for the carboxyl carbon, apocarboxymethyl carbonic anhydrase shows no pH dependence of the chemical shift of its methylene carbon, 51.1 ppm, which is approximately the same chemical shift as is observed for this carbon in the zinc carboxymethyl enzyme above pH 10.

Spin-Coupled ^{13}C NMR Spectra of [2-^{13}C]Carboxymethyl Human Carbonic Anhydrase B. The qualitative relationship between the width of single NMR resonances and the mobility of the groups from which they arise is frequently invoked in discussing mobility of groups in macromolecules. When more quantitative assessments are desired, measurements of spin-lattice relaxation times are often made. However, accumulation of the 5-10 data sets required for accurate characterization of spin-lattice relaxation can be time consuming and in the case of macromolecules with long rotational correlation times changes in T_1 values of single groups are often difficult to assess, since contributions to relaxation may come from intramolecular, intermolecular, and internal rotation contributions to the dipolar mechanism as well as from chemical shift anisotropy. These mechanisms must be separated as well as an a priori calculation made of the contribution to T_1 from overall molecular reorientation before a unique interpretation is possible.

An interesting new method of assessing internal motion of a specific group in a macromolecule has recently been proposed by Prestegard & Grant (1978). These investigators have shown that, for an AX_2 spin system in a large molecule undergoing overall isotropic motion, the relative line widths and peak heights of the coupled spectra are sensitive to the presence of

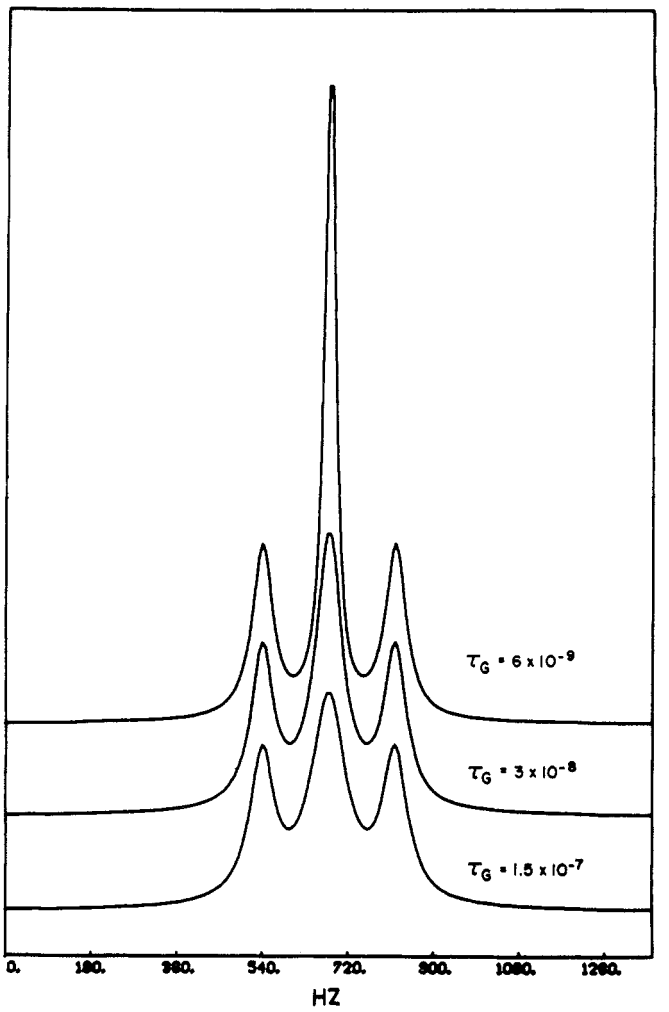

FIGURE 3: Computer simulated spectra for an AX_2 multiplet with overall isotropic reorientation time, τ_r, of 3×10^{-8} s, simultaneously undergoing anisotropic internal motion with correlation times, τ_G, of 6×10^{-9}, 3×10^{-8}, and 1.5×10^{-7} s.

additional internal motion of the group relative to the overall molecular reorientation.[2] The relative line shape changes are independent of changes in the rate of molecular reorientation which can result from changes in solution viscosity, protein aggregation, or temperature. The detailed development of the theory is presented in Prestegard & Grant (1978) and the mathematical development is not appropriate here. The quantitative results of the theory can be illustrated, however, by plotting the theoretical line shapes for the ^{13}C triplet arising from a methylene group in a spherical protein rotating with an overall correlation time, τ_r, of 3×10^{-8} s (appropriate for carbonic anhydrase) and in which internal rotations occur at rates five times faster than, five times slower than, and equal to overall molecular rotation. The theoretical spectra are given in Figure 3 using equations for internal motion about a single axis perpendicular to both C-H vectors.

In addition to the expected narrowing of lines with increasing degree of internal motion, it is obvious that the outer and inner lines change differentially with the central line be-

[2] Internal motion is here defined as significant rotation of the group about a fixed axis moving with the overall reorientation of the protein and characterized by a τ, τ_G, shorter than that characterizing the overall reorientation of the protein, τ_r. Such an operational definition of the internal motion does not exclude internal motion slower than τ_r, but, as indicated in Figure 3, the line shape is relatively less sensitive to slowing of the internal motion such that τ_G is greater than τ_r.

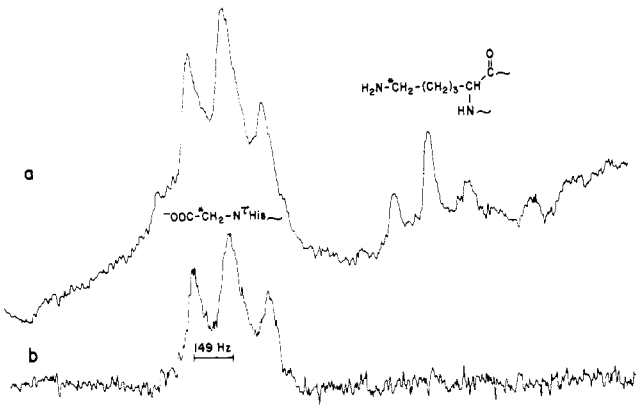

FIGURE 4: Spin-coupled ^{13}C NMR spectrum at 67 MHz of Zn(II) [2-^{13}C]carboxymethyl human carbonic anhydrase B at pH 7.5. (a) Partial spectrum showing the ^{13}C-enriched methylene label and the natural abundance spectrum of the ϵ carbons of the lysyl residues. (b) Difference spectrum of labeled enzyme (a) minus native enzyme. Conditions as in Figure 2.

coming infinitely sharp in the presence of very rapid internal motion. This differential change is purely a function of the presence of internal motion on a time scale short compared with overall molecular rotation. The precise nature of internal motion is not critical to the qualitative assessment of its presence or absence by this method. The same rapid motion limit for multiplet shape is approached as long as the two CH vectors experience equal angular departures from the perpendicular to the assigned rotation axis. For example, if rotation about the methylene C–C bond (rather than about an axis perpendicular to both CH vectors) were to exist, the two C–H vectors would depart from the perpendicular by 20° and spectra would be very similar to those in Figure 3.

If a single ^{13}C triplet could be isolated from the spectrum of a protein the line shapes could potentially be used for an easier and less ambiguous assessment of the degree of internal motion. As shown in Figure 4, isolation of the triplet from the [^{13}C]methylene of the carboxymethylated enzyme is readily obtained and a convenient internal standard happens to be present, the natural abundance ^{13}C resonance of the ϵ-CH_2 groups in the protein (Figure 4).

Since one of the models for the molecular interactions of the carboxymethyl group at the active center of HCAB is the reversible formation of a carboxyl–zinc coordination bond, the rotatory motion of the carboxymethyl group might well be predicted to vary depending on whether it was coordinated or not (see Discussion). In order to assess this possibility, we examined the [^{13}C]methylene triplet under various conditions and used the Prestegard & Grant method for the qualitative detection of changes in the degree of internal motion.

The spectra for the Zn(II) enzyme at pH 7.5, the Zn(II) enzyme at pH 10.6, the Cd(II) enzyme at pH 7.1, and the apocarboxymethyl enzyme at pH 6.3 are shown in Figure 5. Inspection of these spectra does suggest that different degrees of internal motion are present. A tabular way of characterizing the data is given in Table I showing the ratios of peak height and line widths of the outer and inner lines of the triplet in the four enzyme samples. It must be emphasized that such data are purely qualitative, since precision of line widths is limited by the noise and the relative amplitude of the resonances will depend on the magnitude of T_1 for each. Under the same instrumental conditions, however, the trend of the relative peaks heights is a reasonable qualitative indication of the degree of internal motion.

The data on the enzyme show different degrees of internal

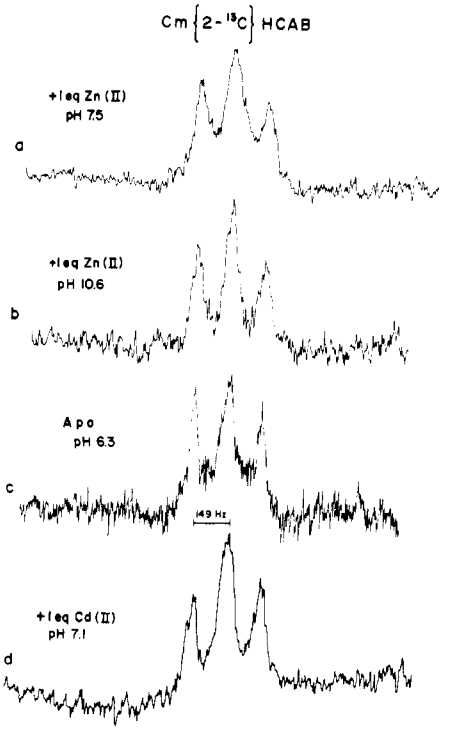

FIGURE 5: Spin-coupled difference ^{13}C NMR spectra at 67 MHz of [2-^{13}C]carboxymethyl human carbonic anhydrase and the corresponding unlabeled human carbonic anhydrases. (a) Zn(II) enzyme at pH 7.5; (b) Zn(II) enzyme at pH 10.6; (c) apoenzyme at pH 6.3; (d) Cd(II) enzyme at pH 7.1. Conditions as in Figure 2.

TABLE I: Approximate Ratios of Peak Heights and Line Widths of the [^{13}C]Methylene Triplet Used in the Qualitative Assessment of the Presence of Internal Motion.

enzyme	ratios of peak heights of outer and inner lines	ratios of line widths of outer and inner lines
Zn(II), pH 7.5	1:1.3:0.8	0.65
Zn(II), pH 10.6	1:1.5:0.9	0.95
Cd(II), pH 7.1	1:1.8:1.1	0.85
ϵ-CH_2, pH 7.5	1:2.1:1	0.85
apoenzyme, pH 6.3	1:1.1:0.9	0.55
theory[a]		
$\tau_G = 1.5 \times 10^{-7}$ s	1:1.3:1	0.70
$\tau_G = 3 \times 10^{-8}$ s	1:1.6:1	0.86
$\tau_G = 6 \times 10^{-9}$ s	1.3.6:1	1.93

[a] Note that the theoretical peak heights are for complete relaxation (recovery) of the line. The specific experimental conditions employed for the enzyme samples do not necessarily achieve this. Thus the peak height ratios of the experimental spectra and the theoretical spectra are not directly comparable, but relative changes through the experimental series remain valid.

motion for the various forms of the carboxymethyl enzyme (Figure 5). The spectrum of the Zn(II) enzyme at low pH is a good example of the case with relatively slow internal rotations. As the pH of the Zn(II) carboxymethyl enzyme is raised to 10.6, the line width of the central line of the methylene carbon triplet narrows, while its amplitude grows indicating a departure from isotropic motion and the onset of a more rapid internal motion. Similar changes are seen when Cd(II) is substituted for Zn(II) at the active site of the carboxymethyl enzyme (Figure 5d). On the other hand, the ^{13}C resonances of the methylene multiplet in the carboxymethyl apoenzyme at pH 6.3 indicate little enhanced internal motion over that of

the Zn(II) enzyme at neutral pH. This may relate to altered structure in the active site cleft of the apoenzyme (see Discussion).

Discussion

The presence of the Zn(II) ion at the active site of human carbonic anhydrase B is required before a pH dependence of the chemical shift of either the [1-^{13}C] or the [2-^{13}C] of carboxymethyl histidine-200 in the carboxymethylated enzyme is observed (Figures 1A and 1B). This finding is not directly accommodated by any of the models proposed for the active center of CmHCAB, since none suggest that protonation of the N$^{\pi}$ of τ-carboxymethyl His-200 should be metal ion dependent (Coleman, 1971, 1975; Strader & Khalifah, 1976; Khalifah et al., 1977). The Zn(II) ion must be involved directly or indirectly in the structure assumed by the carboxymethyl residue. Since the crystal structure of HCAB (Kannan et al., 1975) shows that His-200 is not close enough (~6 Å) to the Zn(II) ion for direct coordination, an interaction of the carboxyl group of the modified residue with the Zn(II), possibly direct coordination, appears likely. This has been a reasonable hypothesis, since the early studies of the carboxymethylation reaction showed the absence of efficient incorportation of the carboxymethyl group into the apoenzyme (Whitney et al., 1967; Bradbury, 1969).

Under conditions where 1 mol of [^{14}C]carboxymethyl group is incorporated into Zn(II) or Co(II) HCAB, none is incorporated into the apoenzyme or Cd(II) HCAB (Coleman, 1971, and unpublished data). This distinction is similar to that observed for the binding of inhibitors, e.g., sulfonamides, known to contribute a donor group to the inner coordination sphere of the metal ion as well as to have additional interactions with the active center cavity (Kannan et al., 1977). [^{3}H]Acetazolamide binds tightly only to the Zn(II) and Co(II) enzymes (Coleman, 1967b). No significant binding of the inhibitor to the apoenzyme or the Cd(II) enzyme is observed until 500-fold higher concentrations of ligand are reached (Coleman, 1967b). Both the Co(II) and Cd(II) enzymes have been extensively characterized. For each ion only a single tight binding site is present on the enzyme and the binding of Co(II) or Cd(II) prevents Zn(II) binding (Coleman, 1967a). Difference electron density maps of the crystal of the Cd(II) enzyme identify the Cd(II) site as identical with that for Zn(II) (Liljas, 1971). An esterase activity has been observed for the Cd(II) (apparent pK_a = 9.1) enzyme (Bauer et al., 1976). A metal-linked ionization with the same pK_a has been identified by the method of perturbed angular correlation of γ rays, i.e., 2 pH units higher than that for the Zn(II) enzyme. Thus the failure of the ^{13}C NMR to indicate any interaction of the carboxymethyl group with the Cd(II) at the active site is compatible with the metal interaction being one of coordination of the carboxyl group to the metal ion, an interaction expected to be much stronger for Zn(II) than for Cd(II). The absence of observable spin–spin coupling between the spin $\frac{1}{2}$ isotope ^{113}Cd(II) at the active site and the [^{13}C]carboxyl carbon is also compatible with this interpretation.

The two inflections observed in the pH titration of the chemical shift of the [^{13}C]-carboxyl of the Zn(II) enzyme (Figure 1A) are qualitatively similar to the two inflections reported by Khalifah et al. (1977), except that the apparent pK_as are higher, pH 7.2 and 9.9, compared with 6.0 and 9.2 reported by the above authors. There are two features of our data which account for this. None of our samples have shown the reversal of the downfield chemical shift of the 1-^{13}C between pH 10 and 11 as reported by Kahlifah et al. (1977) and possibly attributed to incipient gel formation. The continuing downfield chemical shift between pH 10 and 11 in our data accounts for an inflection point of 9.9 rather than 9.2. The chemical shift range between pH 8 and 10 is quite comparable in the two studies. On the other hand, the magnitude of the chemical shift between pH 8 and pH 5 in our study is slightly less than 1 ppm compared with almost 2 ppm in the former study. This derives primarily from our finding of a more downfield chemical shift of the resonance at pH 5.2, 174.2 ppm, rather than ~172.6 ppm reported in the previous study. The chemical shift for the 1-^{13}C of the CmHCAB at pH values between 5 and 8 is extremely sensitive to the ion composition of the medium. Even small amounts of halide, e.g., chloride leaking from a KCl electrode, could cause rather large upfield shifts in the carboxyl resonance in this pH range, as has been documented for iodide by Khalifah et al. (1977). This phenomenon is well documented for the unmodified enzyme and presumably derives predominantly from the anion binding at the active site of the enzyme, an interaction known to get progessively stronger as the pH is lowered. This interaction led us to seek the most unperturbing medium, hence the choice of sodium phosphate and sodium sulfate and the avoidance of electrode contamination (see Methods). Such differences are responsible for the shift of the inflection point toward pH 7. Therefore, we believe that differences in the low pK_a values are primarily due to changes in buffer composition or specific ion effects and that absolute pK_a values do not fundamentally alter conclusions.

From the ^{13}C chemical shifts accompanying titration of the model compound, τ-[1-^{13}C]carboxymethylhistidine, Strader & Khalifah (1976) assigned the lower inflection to the pK_a of the HN$^{\pi}$ of His-200. The complete disappearance of this inflection in the apoenzyme would not be predicted by this assignment, since removal of the metal ion would not a priori be expected to alter the ability of the N$^{\pi}$ to take up a proton. One might argue instead that the best interpretation of the data is that the imidazole ring of His-200 was not titrating and that the carboxyl carbon was being perturbed by two adjacent titrating groups in the active center.

Since the methylene carbon in the model compound, τ-carboxymethylhistidine, undergoes an upfield chemical shift (opposite to that of the carboxyl) when the N$^{\pi}$ deprotonates, observation of the resonance from the 2-^{13}C in the carboxymethyl group during the titration of the enzyme is essential in assigning the observed apparent pK_as of the carboxyl. Only the lower pK_a is observed in the case of the methylene carbon (Figure 2). The direction of the chemical shift is upfield to high pH and the change of chemical shift is from 53 ppm to 51.3 ppm, the same range as observed for titration of the HN$^{\pi}$ in the model compound (Khalifah et al., 1977). The pK_a observed from the methylene titration under our conditions agrees within the error of the measurements with the first pK_a observed from the carboxyl titration (Figures 1 and 2). Both sets of data together strongly suggest that the pK_a of 6.8 to 7.2 in the Zn(II) enzyme results from the titration of the HN$^{\pi}$ of His-200. Since the methylene carbon will be expected to be influenced primarily by electronic changes at the imidazole ring, the observation of the second pK_a at high pH in the ^{13}C NMR titration of the carboxyl carbon, but not of the methylene, suggests that this pK_a corresponds to an ionization process in the vicinity of the carboxyl group. The best candidate appears to be displacement of the carboxyl from the coordination sphere of the metal ion by a competing $^{-}$OH ligand.

Since this hypothesis predicts an unperturbed carboxyl of the carboxymethylhistidine to be present only above pH 10, the normal chemical shift of the deprotonated species, 176 ppm, would not be expected until pH 11, rather than at pH 8

as is observed in the model compound. This is borne out and the chemical shift of the carboxyl carbon in the enzyme remains ~1 ppm (175 ppm) to high field from the shift in the model at pH 8 (176 ppm). This hypothesis also predicts the disappearance of the transition with the high pK_a when the metal is removed. It cannot explain the disappearance of HN$^{\pi}$ ionization as well. Both the carboxyl and methylene carbons in apo-CmHCAB maintain the chemical shifts expected of the deprotonated N$^{\pi}$ species down to pH 6 (Figures 1 and 2). If one retains the original assignment of the low pK_a, then there must be a major shift of the pK_a of the N$^{\pi}$ of carboxymethyl-His-200 when the Zn(II) is removed, to at least pH 5.5. This does not reflect a major change in protein structure, since the original chemical shift and titration behavior are restored when 1 equiv of zinc is added back to the apoenzyme. Such a shift of pK_a to lower pH could result from a change in the environment of the N$^{\pi}$, e.g., placement in a positively charged environment. From the crystal structure, however, there do not seem to be many positively charged residues in the vicinity of His-200; hence a hydrogen bonding interaction may be more likely.

In the unmodified enzyme it is likely to be the HN$^{\tau}$ which titrates, since this side of the ring is clearly exposed to the active site cavity in a position to react with the alkylating agents from solution. The C(2)H of His-200 has been assigned by Campbell et al. (1974) to resonance number 5 in their 270-MHz proton spectrum of the enzyme which titrates with a pK_a of 6.14. Upon carboxymethylation this resonance disappears and no new C(2)H resonance corresponding to His-200 can be observed (Campbell et al., 1974).[3] The HN$^{\pi}$ of His-200 need not have an environment similar to that of the HN$^{\tau}$. In fact the hydroxyl groups of both Thr-199 and Tyr-6 are close enough to the N$^{\pi}$ of His-200 (2.9 and 3.8 Å, respectively) to suggest that hydrogen bonding might occur under some conditions. The N$^{\pi}$ might become hydrogen bonded to one of the nearby hydroxyls when the carboxyl can no longer bind to the metal ion. This could account for the unusually low pK_a in the apo-CmHCAB and in Cd(II)CmHCAB.

There appears to be no alteration of metal binding in the modified enzyme; in fact the Zn(II) ion is slightly more difficult to remove from the carboxymethylated enzyme at neutral pH compared with the native enzyme. Half-times for removal by dipicolinic acid at pH 7 are 2.5 and 2 h, respectively. This finding is compatible with the postulate of an interaction of the carboxyl with the Zn(II) ion. The Cd(II) ion is bound very tightly to the modified enzyme (see Results). Since Zn(II) induces the titration of the HN$^{\pi}$ of His-200, while Cd(II) does not (Figures 1 and 2), it is not unreasonable to postulate that the exposure of the N$^{\pi}$ of carboxymethyl-His-200 so that it titrates above pH 6 has to do with the conformation assumed by the residue when the carboxyl interacts with the Zn(II) ion. Further interpretation of these changes is not possible at present, but it is clear that the carboxymethyl-His-200 must undergo rather dramatic conformational changes induced by the metal ion. Since there is no direct interaction of histidyl-200 with the metal ion and X-ray diffraction studies show no major reorganization of this region of the molecule in the apoenzyme, it seems likely that any conformational change in His-200 of the carboxymethyl enzyme is mediated by a carboxyl–zinc interaction. If this is so, pH-dependent shifts in the chemical shift of the carboxymethyl carbons induced by inhibitors and anions which may compete at either the same or an adjacent coordination site to the carboxyl must be interpreted with caution. Such titrations may represent composite phenomena, anion induced chemical shifts combined with the shift in pK_a of the N$^{\pi}$ of His-200 as the carboxyl–metal ion interaction changes.

One consequence of the above model is that coordination of the carboxyl of the carboxymethyl group to the metal ion would be expected to limit rotational freedom of this group significantly. Conversely conditions such as the high pH or Cd(II) substitution which are predicted to break this coordination bond might be expected to be accompanied by increased rotational motion of the carboxymethyl group. We have used the transverse relaxation method of Prestegard & Grant (1978) applied to the methylene AX_2 spin system to detect changes in internal motion as detailed in Results.

At pH 7.5 the methylene in the Zn(II) carboxymethyl-enzyme shows no evidence of internal motion faster than the overall reorientation of the protein. In contrast, if the pH is raised to 10.6 or Cd(II) is substituted for Zn(II) at pH 7.1, the transverse relaxation indicates the onset of additional internal motion of the methylene carbon (Table I). Since the model based on the chemical shift data suggests that both conditions result in breaking of the coordination bond to the carboxyl, the relaxation data are compatible with this model. On the other hand, the transverse relaxation of the $^{13}CH_2$ in the apoenzyme does not suggest increased rotational freedom of the carboxymethyl group at pH 6.3 (Figure 5). This may indicate considerable change in the active center of the apoenzyme vs. the metalloenzymes. The former metal site must be considerably different in the apoenzyme if for no other reason than there are three additional "free" histidyl residues present (likely to be charged at pH 6.3).

These ^{13}C NMR studies demonstrate the power of a specific NMR-sensitive nucleus located at the active site of an enzyme as a probe of the molecular structure. Not only does it help explore some of the molecular relationships postulated from previous X-ray and solution data, but in the case of the apoenzyme it reveals new aspects of the active center structure not predictable from the crystal structure which must be accounted for in constructing a precise solution structure of the active center of carbonic anhydrase.

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References

Armstrong, J. M., Myers, D. V., Verpoorte, J. A., & Edsall, J. T. (1966) *J. Biol. Chem. 241,* 5137.

Bauer, R., Limkilde, P., & Johansen, J. T. (1976) *Biochemistry 15,* 334.

Bradbury, S. L. (1969) *J. Biol. Chem. 244,* 2002.

Campbell, I. D., Lindskog, S., & White, A. I. (1974) *J. Mol. Biol. 90,* 469.

Coleman, J. E. (1967a) *J. Biol. Chem. 242,* 5212.

Coleman, J. E. (1976b) *Nature (London) 214,* 193.

Coleman, J. E. (1971) *Progress in Bioorganic Chemistry,* (Kaiser, E. T., & Kezdy, F. J., Eds.) Vol. 1, p 159, Interscience, New York, N.Y.

Coleman, J. E. (1975) *Annu. Rev. Pharmacol. 15,* 221.

Harris, M. I., & Coleman, J. E. (1968) *J. Biol. Chem. 243,* 5063.

Hunt, J. B., Rhee, M. J., & Storm, C. B. (1977) *Anal. Biochem. 79,* 614.

[3] We have confirmed this lack of visualization and in addition cannot see the C(2)H or C(4)H protons of His-200 in the 270-MHz proton NMR of the carboxymethylated apoenzyme.

Kannan, K. K., Notstrand, B., Fridborg, K., Lövgren, S., Ohlsson, A., & Petef, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 51.
Khalifah, R. G. (1977) *Biochemistry 16,* 2236.
Khalifah, R. G., Strader, D. J., Bryant, S. H., & Gibson, S. M. (1977) *Biochemistry 16,* 2241.
Liljas, A. (1971) *Crystal Structure Studies of Human Erythrocyte Carbonic Anhydrase C at High Resolution,* Thesis, Acta Universitatis Upsaliensis.
Liljas, A., Kannan, K. K., Bergsten, P.-C., Waara, I., Fridborg, K., Strandberg, B., Carlbom, U., Järup, L., Lövgren, S., & Petef, M. (1972) *Nature (London), New Biol. 235,* 131.
Lindskog, S. (1966) *Biochemistry 6,* 2641.
Lindskog, S., & Coleman, J. E. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 2505.
Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O., & Strandberg, B. (1971) *Enzymes, 3rd Ed. 5,* 587.
Maren, T. M., Rayburn, C. S., & Liddell, N. E. (1976) *Science 191,* 469.
Notstrand, B. (1974) *The High Resolution Structure of Human Carbonic Anhydrase, Form B,* Ph.D. Thesis, University of Uppsala.
Pocker, Y., & Watamori, N. (1971) *Biochemistry 10,* 4843.
Prestegard, J. H., & Grant, D. M. (1978) *J. Am. Chem. Soc. 100,* 4664.
Strader, D. J., & Khalifah, R. G. (1976) *J. Am. Chem. Soc. 98,* 5043.
Whitney, P. L. (1970) *Eur. J. Biochem. 16,* 126.
Whitney, P. L., Nyman, P. O., & Malmstrom, B. G. (1967) *J. Biol. Chem. 242,* 4212.

Complete Amino Acid Sequence of the Major Component Myoglobin from the Humpback Whale, *Megaptera novaeangliae*†

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ABSTRACT: The complete primary structure of the major component myoglobin from the humpback whale, *Megaptera novaeangliae,* was determined by specific cleavage of the protein to obtain large peptides which are readily degraded by the automatic sequencer. Over 80% of the amino acid sequence was established from the three peptides resulting from the cleavage of the apomyoglobin at the two methionine residues with cyanogen bromide along with the four peptides resulting from the cleavage of the acetimidated apomyoglobin at the three arginine residues with trypsin. The further digestion of the central cyanogen bromide peptide with trypsin and *S. aureus* strain V8 protease enabled the determination of the remainder of the covalent structure. This myoglobin differs from that of sperm whale, *Physeter catodon,* at 12 positions, and dwarf sperm whale, *Kogia simus,* at 14 positions, finback whale *Balaenoptera physalus* at 3 positions, minke whale, *Balaenoptera acutorostrata* at 2 positions, and California gray whale *Eschrichtius gibbosus,* at 1 position. All of the substitutions observed in this sequence fit readily into the three-dimensional structure of sperm whale myoglobin.

The complete sequence reported here for the myoglobin from the humpback whale, *Megaptera novaeangliae,* is in total agreement with that found for the first 60 residues by Edman & Begg (1967) in their classical introduction of automated sequencing methodology. The complete amino acid sequence of the myoglobin from Amazon River dolphin (Dwulet et al., 1975), California gray whale (Bogardt et al., 1976), Atlantic bottlenosed dolphin (Jones et al., 1976), arctic minke whale (Lehman et al., 1977), dwarf sperm whale (Dwulet et al., 1977), Pacific common dolphin (Wang et al., 1977), finback whale (DiMarchi et al., 1978a), pilot whale (Jones et al., 1978), and Dall porpoise (Meuth et al., 1978) have been reported. All of these sequences of cetacean myoglobin were determined by automated Edman degradation. This paper reports the application of the peptide fragmentation and analytical procedures that were used in these papers in determining the complete amino acid sequence of the major component myoglobin from the humpback whale. Completion of this sequence extends the number of known cetacean myoglobin sequences to 14. In addition to the above-mentioned proteins, the primary structures of the myoglobins from the Black Sea dolphin (Kluh & Bakardjieva, 1971), common porpoise (Bradshaw & Gurd, 1969; Meuth et al., 1978), sperm whale (Edmundson, 1965; Romero-Herrera & Lehmann, 1974), and killer whale (Castillo et al., 1977) have also been reported.

Experimental Section

Materials

The principal component of humpback whale myoglobin was isolated from muscle tissue as described by Hapner et al. (1968). Phosphate buffer (pH 6.4, 0.1 ionic strength) was used to effect the purification of the crude homogenate on Sephadex CM-50. The homogeneity of the purified myoglobin was shown

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