Histidine-200 Alters Inhibitor Binding in Human Carbonic Anhydrase B. A Carbon-13 Nuclear Magnetic Resonance Identification†

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ABSTRACT: We have previously prepared ^{13}C-enriched N^{τ}-carboxymethylhistidine-200 human carbonic anhydrase B (CmHCAB) by reacting the native enzyme with 90% [1-^{13}C]bromoacetate (Strader, D. J., and Khalifah, R. G. (1976), *J. Am. Chem. Soc. 98,* 5043). The ^{13}C nuclear magnetic resonance signal of the enriched carboxylate of CmHCAB proved sensitive to active-site events, permitting, among other things, the determination of the *microscopic* pK_a of the modified histidine. This report extends the study to the complexes of CmHCAB with the inhibitors iodide and azide. It is found that the pK_a of histidine-200 is significantly increased when these inhibitors bind. A *quantitative* comparison of the iodide-induced pK_a shift with literature data (Whitney, P. L., and Brandt, H. (1976), *J. Biol. Chem. 251,* 3862) showing that the binding of iodide is influenced by the ionization of an active-site group of pK_a 6.1 allowed the clear identification of histidine-200 as the perturbing group. Other important implications of the magnetic resonance results are also discussed.

The mechanism of inhibitor binding to carbonic anhydrase (carbonate hydro-lyase EC 4.2.1.1) has been extensively studied in recent years (Maren, 1967; King and Burgen, 1970; Lindskog et al., 1971; Taylor and Burgen, 1971; Taylor et al., 1970a,b, 1971; Lanir and Navon, 1974; Coleman, 1965, 1975). The pH dependence of the binding of anionic inhibitors is known to depend on the ionization of the so-called catalytically essential group controlling the enzyme activity, such that the basic inhibitors bind only when this group is formally in its acid form (Lindskog et al., 1971). A recent study on the low specific activity isozyme B of human carbonic anhydrase (HCAB[1]) demonstrates for the first time the influence of a second active-site group of pK_a 6.1 whose ionization significantly alters the affinity for halide anionic inhibitors (Whitney and Brandt, 1976). The nature of that study (determination of the pH dependence of the K_i) did not permit the identification of this second group, although it seemed likely that it should be one of several histidines known to occur in the active site from x-ray crystallographic (Kannan et al., 1975) and chemical modification (Whitney et al., 1967) work. It should be added that the identity of the catalytically essential group is itself the subject of much debate, with most evidence implicating a metal-coordinated ligand of the essential zinc atom of this metalloenzyme (Lindskog and Coleman, 1973; Campbell et al., 1974; 1975; Lindskog et al., 1971; Khalifah et al., 1977).

The elucidation of the detailed role of active-site groups in the mechanism of inhibitor binding and catalysis requires the ability to observe and monitor their individual properties. Most previous work has centered on the role of the essential metal and has either made use of the spectroscopically useful substitution of Co^{2+} for zinc at the active site (Lindskog, 1966; Coleman, 1965; Coleman and Coleman, 1972) or has focused on the properties of the inhibitors or substrates (Coleman, 1968; Taylor et al., 1971; Feeny et al., 1973; Yeagle et al., 1975). However, we have recently succeeded in introducing a ^{13}C nuclear magnetic resonance (^{13}C NMR) "probe" into the active site of HCAB by reacting the enzyme with 90% [1-^{13}C]bromoacetate under conditions that result in exclusive N^{τ}-carboxymethylation of histidine-200 (Strader and Khalifah, 1976; Khalifah and Strader, 1977; Khalifah et al., 1977). The ^{13}C NMR signal of the covalently attached, enriched carboxylate proved very sensitive to ionizations of nearby active-site groups, permitting the determination of their microscopic pK_a values. The present report is an extension of these studies to include complexes of CmHCAB with anionic inhibitors of the enzyme. The results are complementary to the above-mentioned work of Whitney and Brandt (1976) such that the combined data unambiguously identify histidine-200 as the second active-site group (pK_a 6.1) whose ionization alters inhibitor binding. Other implications of the data are also discussed.

Materials and Methods

Human carbonic anhydrase B was prepared by a single-column, single-pass affinity chromatography method developed in our laboratory (Khalifah et al., 1977). Enriched CmHCAB was prepared by reaction of HCAB with 90% [1-^{13}C]bromoacetate as previously described (Strader and Khalifah, 1976). The carboxymethylation occurs exclusively at N^{τ} of histidine-200 of the active site (Andersson et al., 1969, 1972; Bradbury, 1969a,b). The enriched bromoacetate was purchased from Koch Isotopes. ^{13}C NMR spectra were run on a JEOL PFT-100/EC 100 Fourier transform spectrometer operating at a carbon frequency of 25.15 MHz. Sample tubes were of the 10-mm flat-bottom type and were used in conjunction with Teflon vortex plugs. Aliquots of CmHCAB (2–6 mM) of about 0.6–1.0 mL were individually dialyzed against 250 mL of Tris or Bistris buffer at the appropriate pH (adjusted with H_2SO_4) and containing the desired concentration of inhibitor. After overnight dialysis at room temperature, the

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[1] Abbreviations used are: HCAB, human erythrocyte carbonic anhydrase isozyme B; CmHCAB, N^{τ}-carboxymethylhistidine-200 human carbonic anhydrase B; τ-CmHis, N^{τ}-carboxymethyl-L-histidine (these follow the recommended notation of the IUPAC–IUB ICN (1975); N^{τ} is frequently referred to as the 3′ nitrogen according to common biochemical usage or the 1 nitrogen according to usage preferred by chemists); Tris, tris(hydroxymethyl)aminomethane; Bistris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Me_4Si, tetramethylsilane; EDTA, ethylenediaminetetraacetate.

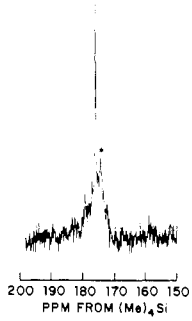

FIGURE 1: A 25.15 MHz ^{13}C NMR spectrum of enriched CmHCAB in the presence of 0.25 M KI inhibitor at pH 7.91 and 25 °C. Only the carbonyl carbon region is shown. The large narrow resonance is that due to the covalently attached enriched carboxylate. The tallest peak of the natural abundance background of the enzyme is marked with the asterisk.

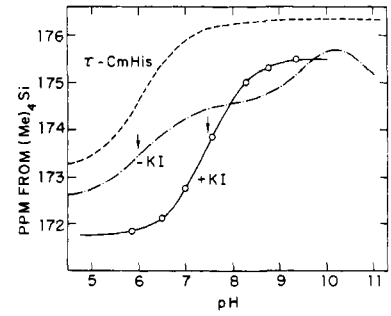

FIGURE 2: pH dependence of the ^{13}C NMR signal of enriched CmHCAB in the presence of 0.5 M KI at 25 °C (experimental points). Solid line represents a theoretical fit (see text). The titration behavior in the absence of KI (- - -) is shown for comparison, as is the titration curve for the corresponding carboxylate of τ-CmHis (- - -). Buffers were 0.05 M Tris or Bistris-sulfate.

samples were taken out and augmented with two drops of D_2O for deuterium locking and 2 μL of dioxane for internal chemical shift referencing. All reported chemical shifts are in parts per million downfield from Me_4Si and were converted to this scale by assuming that dioxane is 67.40-ppm downfield from Me_4Si (Levy and Cargioli, 1972). The pH of each sample was read immediately after each spectrum was taken and was usually within 0.02 of the pH of the dialysis buffer. Spectra were the average of 550–7500 transients using a 0.9- or 1.7-s recycle time and a 55° flip angle. An 8K of zero-fill was frequently employed with 8K data points to improve peak definition. All spectra and measurements were taken at 25 °C.

Results

^{13}C NMR Spectrum of Inhibited CmHCAB. The ^{13}C NMR resonance of the enriched carboxylate of CmHCAB in the absence of inhibitors is easily detected over the natural abundance background signals of some 314 other carbonyl and carboxyl carbons in this 29 000 molecular weight enzyme (Khalifah and Strader, 1977; Khalifah et al., 1977). Although the signal shows considerable exchange broadening in the presence of certain inhibitors, notably sulfonamides (Strader and Khalifah, 1976), this is not the case with azide and iodide inhibitors. A typical carbonyl carbon region spectrum is shown in Figure 1 for the iodide complex of enriched CmHCAB. The large, sharp signal close to 175 ppm is that of the single-enriched carbon, while the signal marked with the asterisk represents the tallest peak of the natural abundance background. There was thus no ambiguity in following the position of this signal over the full range of pH covered here and at much lower signal-to-noise ratios than shown in Figure 1. The azide complex behaved very similarly.

^{13}C NMR Titration Shifts of the CmHCAB–Iodide Complex. A ^{13}C NMR titration of the enriched carboxyl of CmHCAB was carried out between pH 5.8 and 9.2 in the presence of 0.5 M KI (experimental points in Figure 2). The dissociation constant (K_i) for iodide inhibition of CmHCAB is known from the work of Whitney and Brandt (1976). It increases (weaker inhibition) with increasing pH, being approximately 25 mM at pH 9.2. We thus estimate that the enzyme is better than 95% inhibited throughout the range covered and that we are observing the pH dependence of the chemical shift of the carboxylate in the *complex* of CmHCAB with the iodide inhibitor. It is apparent that the resonance is perturbed by the ionization of a nearby group. The data can be fit (solid line of Figure 2) by assuming that the observed chemical shift (δ_{obsd}) is a weighted average of the shifts of the two states in

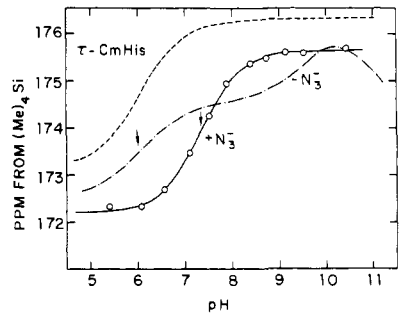

FIGURE 3: pH dependence of the ^{13}C NMR signal of enriched CmHCAB in the presence of NaN_3 at 25 °C (experimental points). The solid line represents a theoretical fit (see text). The dashed and dot-dash curves are as in Figure 2.

which the perturbing group is protonated (δ^+) or deprotonated (δ°):

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

where f^+ and f° are the fractions of molecules in each ionization state at any pH. These are readily calculated to be:

$$f^+ = ([H^+]/K_a^i)(1 + [H^+]/K_a^i)^{-1}$$

and

$$f^\circ = (1 + [H^+]/K_a^i)^{-1}$$

where K_a^i is the ionization constant of the perturbing group in the *complex* of CmHCAB with iodide. The best fit for the iodide data were obtained by assuming the following values: δ^+ = 171.75 ppm, δ° = 175.50 ppm, and pK_a^i = 7.45. The average deviation of the points from the curve was 0.02 ppm, well within the estimated error of 0.05 ppm in measuring the chemical shifts. The magnitude of the titration shift ($\delta^\circ - \delta^+$) then becomes 3.8 ppm.

^{13}C NMR Titration Shifts of the CmHCAB–Azide Complex. A similar ^{13}C NMR titration study was carried out on enriched CmHCAB in the presence of NaN_3 (experimental points in Figure 3). The data were taken at 0.2 M N_3^-, except for the point at pH 7.90 (0.1 M N_3^-) and that at pH 5.39 (2 mM N_3^-), although the ionic strength was maintained close to 0.2. Accurate K_i values have not been reported in the literature for CmHCAB, but using the Wilbur-Anderson changing-pH assay (Rickli et al., 1964) we estimate that azide is a more powerful inhibitor than iodide by a full order of magnitude. Consequently, with the possible exception of the point at pH 5.39, we estimate that we are observing the carboxylate

resonance in the azide complex of CmHCAB. The observed pH dependence is rather similar to the iodide complex. The data could be fit assuming perturbation by a single group with pK_a^i of 7.34, δ^+ = 172.20 ppm, and δ° = 175.64 ppm. The average deviation of the points from the best-fit curve (solid line of Figure 3) is 0.04 ppm, also within experimental error. The overall titration shift ($\delta^\circ - \delta^+$) is thus about 3.4 ppm.

Discussion

The ^{13}C signal from the single-enriched carboxylate of CmHCAB has proven to be easily observable and assignable in both the uninhibited enzyme (Strader and Khalifah, 1976; Khalifah and Strader, 1977; Khalifah et al., 1977) and its complexes with anionic inhibitors (Figure 1). However, the pH dependence of the resonance is very different in the presence of inhibitors. In free CmHCAB, the chemical shift has been observed to be influenced by three nearby ionizations, leading to inflections at pH 6.0, 9.2, and $\geq$10.7, as can be seen above in the (- · -) curves in Figures 2 or 3 (Khalifah and Strader, 1977). The pK_a 6.0 inflection has been assigned to the ionization of histidine-200 itself (protonation at N^π), whereas the pK_a 9.2 inflection has been attributed to the ionization of the so-called catalytically essential group, presumably a water-hydroxyl ligand of the zinc (Khalifah et al., 1977; Strader and Khalifah, 1976).

The ^{13}C NMR titration curves of the iodide and azide complexes of CmHCAB (Figures 2 and 3) show, by contrast, the influence of only a single ionizing group in the pH range covered. The pK_a values differ slightly in the two complexes (7.45 and 7.34) and are distinct from any of those seen in the titration of free CmHCAB. The magnitudes of the titration shifts are 3.4–3.8 ppm, much larger than those of CmHCAB (- · - curves of Figures 2 and 3), but rather close to the 3.1-ppm titration shift seen (Khalifah et al., 1977) for the corresponding carboxyl of τ-CmHis upon ionization of its imidazole group (- - - curve in Figure 2 or 3). We can thus confidently attribute the pH dependence in the complexes to the ionization of the carboxymethylated histidine-200 and extract microscopic pK_a values of 7.45 and 7.34 for this residue in the iodide and azide complexes, respectively. This represents a substantial inhibitor-induced increase in the pK_a from 6.00 in free CmHCAB.

The absence of a higher inflection due to the ionization of the catalytically essential group may be a consequence of the inability to extend the measurements to a higher pH in these complexes. This appears rather unlikely, however. There is substantial evidence that the inhibitors of this type do not bind to the enzyme when the catalytically essential ionizing group is deprotonated at high pH. This comes from studies of the pH dependence of inhibitor binding (Lindskog, 1966; Whitney and Brandt, 1976), as well as from inhibitor suppression of pH-dependent changes in the visible spectrum of Co^{2+}-substituted carbonic anhydrase (Lindskog, 1966; Coleman, 1965; Whitney and Brandt, 1976). In addition, x-ray crystallographic studies have established that inhibitors bind at the zinc, displacing the water ligand in most cases (Bergstén et al., 1972).

The magnitude of the ^{13}C NMR titration shifts due to histidine-200 ionization in the inhibitor complexes of CmHCAB and the mode of binding of the inhibitors at the zinc's fourth (water) coordination site both provide excellent support for our previous proposal (Khalifah et al., 1977; Khalifah and Strader, 1977) of a pH-dependent interaction between the observed carboxylate and the zinc or its aquo ligand. This proposal was based on an interpretation of the difference in (1) the absolute chemical shift of the carboxylate and (2) in the magnitude of its titration shift upon imidazole ring ionization when free CmHCAB is compared with the model compound τ-CmHis (- · - and - - - curves, respectively, in Figures 2 or 3). These differences could *both* result from an upfield shifting "abnormal" environment of the carboxylate at the pH 8 plateau, but not at pH 5 or 10, and such an upfield shift could be caused by the approach of the carboxylate towards a positively charged group (Batchelor, 1975; Batchelor et al., 1975) such as the zinc or its water ligand.[2] It is reasonable to expect then that binding of inhibitors at the zinc should disrupt such an interaction and restore the ^{13}C NMR titration shift associated with histidine-200 ionization to a more normal value characteristic of the model compound. The titration shifts seen in the iodide and azide complexes are in excellent agreement with this expectation and thus provide support for the proposed carboxylate–zinc or carboxylate–water ligand interaction in CmHCAB when histidine-200 is deprotonated and the zinc-hydroxyl ligand is (formally) protonated. Such an interaction has important mechanistic implications (Khalifah et al., 1977) and may account, for example, for the much higher pK_a for the zinc–water ionization in CmHCAB compared to HCAB. It is interesting that Coleman (1975) has previously suggested a *direct* carboxylate–zinc ligation in CmHCAB and discussed its consequences, although experimental evidence has been lacking until now.

The ^{13}C NMR data on the iodide complex provide a clear interpretation of the results of Whitney and Brandt (1976) on the pH dependence of iodide binding to CmHCAB mentioned above in the Introduction. These authors found that the pH dependence of the inhibition constant, K_i, is controlled by the ionization of two enzyme groups with pK_a values of about 9.0 and 6.1 in free CmHCAB. The former is presumably the catalytically essential group whose deprotonation abolishes inhibition, while the latter is a previously unsuspected active-site group whose deprotonation decreases the affinity for iodide by approximately an order of magnitude (K_i increases from 0.6 to 10 mM). Since we have previously found a pK_a of 6.0 for histidine-200 in free CmHCAB (Strader and Khalifah, 1976; Khalifah and Strader, 1977; Khalifah et. al., 1977), and this pK_a is substantially altered in the inhibitor complexes, histidine-200 becomes a prime candidate for the role of the second perturbing active-site group. This hypothesis is amenable to a *quantitative* verification, as the analysis below shows, since a reciprocal effect should exist between the inhibitor alteration of the pK_a and the ionization effects on the pK_i.

A general scheme for the linkage between ionization and inhibitor binding is shown in Figure 4. The X denotes the catalytically essential group and the Y refers to the second perturbing group, the notation being similar to that of Whitney and Brandt (1976). We can ignore the species $HYEX^-$, since the two pK_a values of the X and Y groups are so far apart (9.0–9.2 and 6.0–6.1, respectively). We can also ignore all species containing $X^-{\cdot}I$, since the inhibitor binding is abolished

[2] The former of these two possibilities, i.e. *direct* ligation of the carboxyl to the zinc, was previously considered less likely (Strader and Khalifah, 1976; Khalifah et al., 1977), primarily on the basis of visible spectral studies of Co^{2+}-substituted CmHCAB and its complxes with acetates (Whitney, 1970; Taylor et al., 1970b). The original spectral interpretations have been recently challenged by Coleman (1975) who does not find the data inconsistent with direct ligation. The abnormal chemical shift that we see at pH 8 relative to the model compound τ-CmHis is very consistent with direct ligation. For example, we find that zinc coordination induces an upfield 1.4-ppm shift in the carboxylates of EDTA (see also Howarth et al., 1974).

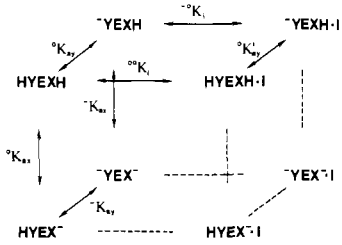

FIGURE 4: Equilibria for inhibitor (I) binding to CmHCAB when two ionizing groups (X and Y) on the enzyme affect the binding. All constants are dissociation constants.

upon deprotonation of the group with pK_a 9.0–9.2 (Whitney and Brandt, 1976). The scheme then simplifies to the following relevant equilibria:

$$\begin{array}{ccc} \text{HYEXH} & \overset{^{\circ}K_{ay}}{\rightleftharpoons} & {}^{-}\text{YEXH} \overset{^{-}K_{ax}}{\rightleftharpoons} {}^{-}\text{YEX}^{-} \\ {\scriptstyle {}^{\circ\circ}K_i}\Updownarrow & & \Updownarrow{\scriptstyle {}^{-\circ}K_i} \\ \text{HYEXH·I} & \overset{^{\circ}K^{i}_{ay}}{\rightleftharpoons} & {}^{-}\text{YEXH·I} \end{array}$$

Note that the K_a's are ionization constants and the K_i's are inhibitor dissociation constants. The latter values are available for iodide inhibition from the study of Whitney and Brandt and are given in Table I along with the ionization constants for the catalytically essential group and for histidine-200 in free and iodide-inhibited CmHCAB. The above scheme obviously requires the following linkage relation to hold:

$$^{\circ\circ}K_i \; {}^{\circ}K_{ay} = {}^{\circ}K_{ay}{}^{i} \; {}^{-\circ}K_i$$

We can express this in terms of the pK values and rearrange to get:

$$p^{\circ}K_{ay}{}^{i} - p^{\circ}K_{ay} = p^{\circ\circ}K_i - p^{-\circ}K_i$$

The right-hand side is equal to 1.22 from the values in Table I and this should thus be equal to the difference in pK_a values of the second perturbing group in the presence and absence of bound iodide. Our present results for histidine-200 show that a ΔpK_a of 1.45 is produced by iodide binding, in satisfactory agreement with the requirements for this residue to be the perturbing group. The agreement is even closer when it is noted that the pK_a of histidine-200 in the presence of iodide was obtained at an ionic strength of 0.5 M, whereas the pK_a in free CmHCAB was obtained at an ionic strength of 0.2 M. Since increasing the ionic strength will tend to raise the pK_a of an imidazole group (Edsall and Wyman, 1958), the ΔpK_a will be correspondingly less than 1.45 when this is taken into account.[3]

The above analysis and the measured pK_a of 6.0 for histidine-200 in free CmHCAB leaves little doubt that this residue is the group that perturbs inhibitor binding in CmHCAB. This situation is not limited to the binding of iodide, since Whitney and Brandt (1976) reported similar effects on the inhibition by Br^- and Cl^-, while we have reported here that azide causes an alteration in the pK_a of histidine-200. In fact, we have preliminary evidence (unpublished observations) that substantially larger pK_a shifts occur in the presence of certain sulfonamide inhibitors. The molecular basis for these reciprocal

TABLE I: Dissociation Constants Relevant to pH Dependence of Inhibitor Binding in CmHCAB at 25 °C.

Equilibrium step[a]	Dissociation constant[a]	pK
Binding of iodide to fully protonated CmHCAB (HYEXH)	$^{\circ\circ}K_i$	3.22[b]
Binding of iodide to CmHCAB when only pK_a 6.1 group is deprotonated ($^{-}$YEXH)	$^{-\circ}K_i$	2.00[b]
Ionization of catalytically essential group (XH) in free CmHCAB	$^{-}K_{ay}$	9.2[c,d]
Ionization of histidine-200 (presumably YH) in free CmHCAB	$^{\circ}K_{ay}$	6.0[d]
Ionization of histidine-200 (presumably YH) in iodide complex of CmHCAB	$^{\circ}K_{ay}{}^{i}$	7.45[e]

[a] See scheme of Figure 4. [b] From data of Whitney and Brandt (1976) at 0.2 ionic strength. [c] This refers to the pK_a of the spectral change of Co^{2+}-CmHCAB at an ionic strength 0.075 (Whitney, 1970). [d] From ^{13}C NMR titration shifts of enriched CmHCAB at an ionic strength of 0.2 (Strader and Khalifah, 1976). [e] Results of this study (Figure 2) at an ionic strength of ~0.5.

effects may lie in a stabilizing interaction between a protonated histidine-200 and a negatively charged bound inhibitor. This could be of the form of a hydrogen bond between the proton on N^{π} and the inhibitor, possibly with the intermediacy of a solvent molecule or a protein side chain, such as the hydroxyl of threonine-199 inferred to be nearby in the crystal structure of the enzyme (Kannan et al., 1975; Notstrand et al., 1975).

It is not obvious why such an interaction should not occur also in HCAB or its other derivatives. Whitney and Brandt (1976) have presented similar data on the inhibition of HCAB carboxamidomethylated at N^{τ} of histidine-200 (CamHCAB) where a perturbing group of pK_a ~ 6.1 also influences the binding. Note that, in this derivative, the pK_a of the catalytically essential group is decreased to 8.2. They have also discussed evidence for perturbations by a similar group in HCAB, where the pK_a value of the catalytically essential group drops further to about 7.3–7.5, making detection of the perturbation of the pK_a 6.1 group much more difficult.[4]

The interaction of histidine-200 and the anionic inhibitors may have more important implications that extend to the mechanism of catalysis of CO_2 hydration of HCAB. It is commonly suggested that HCO_3^-, the reverse substrate or product of CO_2 hydration, may bind to the same site as the anions, so that it is reasonable to expect its binding to be influenced by histidine-200. This may well explain the observation that the pH dependence of the catalysis kinetics of HCAB (Khalifah, 1971) cannot be fit to a model in which only one ionizing group influences the kinetics. This is in contrast to the kinetics of the high-activity C isozyme (Khalifah, 1971) in which histidine-200 is replaced by a threonine (Henderson et al., 1976). In addition, the CO_2 hydration kinetics of CmHCAB are considerably complex in their pH dependence (Khalifah and Edsall, 1972). Previous rationalization of the kinetics of this derivative did not take into account the possible influence of a second active-site group aside from the catalytically essential one, so that a reassessment of the analysis

[3] We have, in fact, measured the shift in the presence of only 0.25 M KI at pH 7.91 and found it to be off the best-fit titration curve of Figure 2 (solid line) by a downfield 0.40 ppm. This translates to a decrease in the pK_a (left shift of the 0.5 M KI curve) by 0.20 pH unit in going to the lower ionic strength.

[4] Whitney and Brandt (1976) also reported that a group with pK_a 6.1 perturbs the visible spectrum of uninhibited Co^{2+}-CmHCAB and Co^{2+}-HCAB. This may be supportive of our proposal[2] (Khalifah et al., 1977) of the movement of the carboxylate away from the zinc vicinity when histidine-200 is protonated.

may be necessary when the pK_a of histidine-200 is determined in the presence of HCO_3^- (work in progress).

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