Interaction of the Unique Competitive Inhibitor Imidazole with Human Carbonic Anhydrase B†

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ABSTRACT: Imidazole was previously found to be unique among the inhibitors of human carbonic anhydrase B (HCAB) in that it binds competitively with the CO_2 substrate (Khalifah, R. G. (1971), *J. Biol. Chem. 246,* 2561). We report here an aromatic ultraviolet difference spectral study of its interaction with HCAB and compare it with a variety of other inhibitors. Imidazole is found to be unique in that: (1) it generates a different spectrum upon binding that is also much suppressed in intensity; (2) its affinity for HCAB is maximal at *high* pH, being abolished upon its protonation and being independent of active-site ionizations. Imidazole differs from CO_2 in that it binds competitively with the anionic inhibitor iodide. The unique properties of imidazole binding are consistent with the recently determined crystal structure of its complex with HCAB showing it to bind as a weak and distant fifth ligand of the essential zinc atom, rather than displacing the solvent molecule in the fourth ligand position (Kannan, K. K., Petef, M., Fridborg, K., Cid-Dresdner, H., and Lövgren, S. (1977), *FEBS Lett. 73,* 115).

Human erythrocyte carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a monomeric zinc metalloenzyme of molecular weight 29 000 that occurs in the form of two major isozymes designated HCAB[1] and HCAC (Lindskog et al., 1971). These isozymes are homologous, showing approximately 60% identity in their amino acid sequences (Andersson et al., 1972; Henderson et al., 1976). They also have similar three-dimensional structures that have been elucidated by x-ray crystallography (Notstrand et al., 1975). Nevertheless, they exhibit important differences, such as in their level of expression in the erythrocyte and in their catalytic specific activity (cf. Lindskog et al., 1971). In addition, their inhibition by various anions and sulfonamides shows quantitative differences (Maren, 1967) that could conceivably be of physiological significance (Maren et al., 1976).

A most unique and surprising inhibitor of the low specific activity HCAB is imidazole (Khalifah, 1971). This inhibitor, aside from having almost no affinity for the high-activity isozyme HCAC (Khalifah, 1971; Kernohan, 1964), is the only known competitive inhibitor of the CO_2 substrate in HCAB. No other competitive inhibitor of CO_2 is known to either isozyme. Its importance has prompted us to study its interaction with HCAB, as described in this report. We discuss our results in terms of the recently determined crystal structure of the imidazole complex of HCAB that showed an unusual mode of binding at the active site (Kannan et al., 1977).

Materials and Methods

Preparation of HCAB. The enzyme was prepared from the hemolysate of freshly outdated erythrocytes kindly donated by the Blood Bank of the University of Virginia Hospital. The removal of hemoglobin and the resolution of isozymes was then carried out either by DEAE-Sephadex chromatography (Armstrong et al., 1966) or, more recently, by the affinity chromatography method developed in our laboratory (Khalifah et al., 1977). Enzyme concentrations were determined from the absorbance at 280 nm (Lindskog et al., 1971).

Chemicals. Imidazole was obtained from Sigma and was further purified by two recrystallizations from acetone–petroleum ether. Sodium cyanate was from Aldrich Chemicals, while Tris, Bistris, and sodium azide were from Sigma. DEAE-Sephadex A-50 was a product of Pharmacia. Tryptophan, tyrosine, and phenylalanine were gifts from Dr. R. B. Martin. All other chemicals were Analytical Reagent grade.

Spectroscopy. All the reported difference spectra were obtained on a GCA/McPherson EU-707D double-beam filter-grating spectrophotometer having a linear response up to an optical density of 3.0. Its performance and calibrations were continuously monitored and checked following the general guidelines of Donovan (1969). Difference spectra were obtained using tandem cells (Hellma), each compartment of the split cell having an optical pathlength of 0.438 cm. Identical volumes of 1.0 mL of enzyme and inhibitor were pipetted separately into each compartment. A difference spectrum was generated by subtracting the spectrum taken with the contents mixed from the spectrum taken before mixing. Thus, a *positive* difference indicates higher absorbance in the *uninhibited* enzyme. Subtraction was done automatically by feeding the output signals from the spectrophotometer into a Varian CAT computer, the first scan (unmixed) with positive polarity, or addition sense, and the second scan (mixed) with negative polarity, or subtraction sense. Precise wavelength synchronization was achieved by using the digital pulses of the spectrophotometer, which advance the monochromator in discrete increments, to advance the CAT memory channel by channel. The reference sample was a similar enzyme solution that provides sufficient blanking to use the 0–0.1 A full-scale capability, 0.1 A being equivalent then to 1 V output to the CAT. The reference was untouched during the two scans before and after mixing. This procedure caused automatic subtraction of any baseline nonlinearity due to imperfect matching of the beam optics. After subtraction, the memory contents of the CAT were then written out on an X–Y recorder (Houston Instrument Ominigraphic 2000). All scans covered the spectral

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[1] Abbreviations used are: HCAB and HCAC, human carbonic anhydrase isozymes B and C; Tris, tris(hydroxymethyl)aminomethane; Bistris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; DEAE, diethylaminoethyl; NMR, nuclear magnetic resonance.

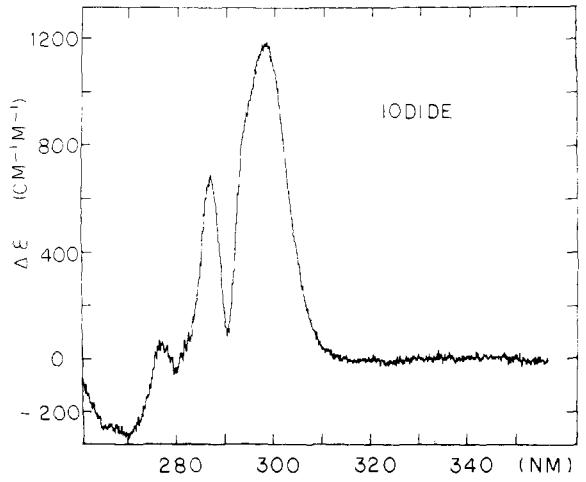

FIGURE 1: Difference spectrum produced by the inhibition of HCAB by iodide at pH 7.3 at a saturating concentration (20 mM). Positive difference indicates higher absorbance in the *uninhibited* enzyme. [HCAB] was 4.5 × 10^{-5} M after mixing.

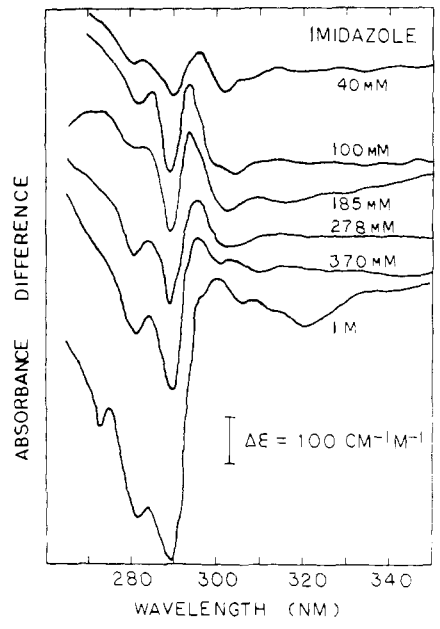

FIGURE 2: Difference spectra generated by adding varying amounts of imidazole, as indicated, to HCAB at pH 7.3 and 0.2 ionic strength. Spectra are arbitrarily offset for clarity, but zero can be located at 350 nm where neither inhibitor nor enzyme absorbs. Spectrum has same sense as Figure 1 but note the difference in ordinate scale. [HCAB] was 4.4 × 10^{-5} M.

TABLE I: Difference Spectral Intensities Produced by Inhibitors of HCAB.[a]

Inhibitor	Concn[b] (mM)	$\Delta\epsilon_{299}$[c] (cm^{-1} M^{-1})
NaOCN	2.2	960
KSCN	24	990
Ethyl carbamate	220	910
$NaNO_3$	300.0	1060
$NaHCO_3$	130[d]	1000
NaF[e]	500	~0
NaCl	250[d]	940
KBr	115[d]	1080
KI	10	1190
NaN_3	1.5	900
Imidazole	( )	200[f]

[a] Measurements at 25 °C in pH 7.3 Bistris-sulfate buffers. [b] Concentrations were at least 20-fold the literature K_i unless noted otherwise. [c] Positive values indicate higher absorbance in the *uninhibited* enzyme. [d] Concentrations are fivefold the K_i. Higher concentrations caused an anomalous *decrease* in $\Delta\epsilon$. [e] Control, since NaF is not known to significantly inhibit the enzyme. [f] Value obtained by curve fitting (see Discussion in text) and peak is at 294–295 nm rather than 299 nm.

range 260.00–362.40 nm. Difference spectra errors due to pipetting errors or drift were easily recognizable.

Results

Difference Spectra of Inhibitors Other Than Imidazole. We examined for comparative purposes the aromatic difference spectra generated at pH 7.3 by a variety of HCAB inhibitors. For the spectral range of 260–360 nm, all the other inhibitors generated a nearly identical difference spectra dominated by a positive difference peak (inhibitor-induced blue shift) at 298–299 nm, a smaller peak at 286–287 nm, and a minor "peak" at 276–277 nm, as shown for iodide in Figure 1. Besides the constancy of the appearance of the difference spectrum, the $\Delta\epsilon$ at 298–299 nm for the fully inhibited complexes were almost invariably in the range of 900–1200 cm^{-1} M^{-1} (Table I), despite differences in the structure, size, and polarity of the inhibitors. These observations also apply to the difference spectra generated by sulfonamides and some other anions that were studied by King and Burgen (1970). It should be emphasized that the difference spectra are due to active-site binding and do not originate from nonspecific solvent-perturbation considerations. Solvent-perturbation spectra are almost invariably associated with a red shift (Donovan, 1969). The effects we see show saturation[2] behavior as a function of inhibitor concentration, the dependence following a simple binding isotherm (see below). In addition, substances such as F^- that are not inhibitory do not produce a difference spectrum at similar concentrations.

Difference Spectrum Generated by Imidazole. The difference spectra generated by increasing concentrations of imidazole at pH 7.3 are shown in Figure 2. It is apparent that the behavior is more complex than expected for simple binding. At low imidazole concentrations comparable to the reported dissociation constant (Khalifah, 1971), a small positive (blue shifted) difference spectrum is generated that differs from that produced by other inhibitors. The spectrum has a maximum at 294–295 nm and decreases to zero around 290 and 300 nm. High concentrations of imidazole, however, produce a red-shift (negative) difference spectrum, with a negative peak at ~290 nm. This latter difference spectrum somewhat resembles solvent-perturbation difference spectra (Lindskog and Nilsson, 1973) of HCAB. We have plotted the peak-to-trough difference, i.e. $\Delta(\Delta\epsilon) \equiv \Delta\epsilon_{289} - \Delta\epsilon_{295}$, as shown by the experimental points in Figure 3. The continuous curve is a theoretical fit discussed below (see Discussion).

Imidazole as a Solvent Perturbant of Tryptophan and Tyrosine. In view of the above results, we examined the effects of imidazole on the amino acids tryptophan and tyrosine. In both cases, a red-shift (negative) difference spectrum was produced. The tryptophan difference spectrum was highly similar to that of Figure 1, although of opposite sense and with different splittings of the three maxima (292, 284, and 274 nm). The tyrosine difference spectrum had maxima at 286 and 278 nm, typical of tyrosine perturbation difference spectra (Donovan, 1969). In both cases, the $\Delta\epsilon$ varied linearly with

[2] A subsequent anomalous *decrease* in the magnitude of $\Delta\epsilon_{299}$ was observed with some anions, especially the halides, at inhibitor concentrations above 0.5 M.

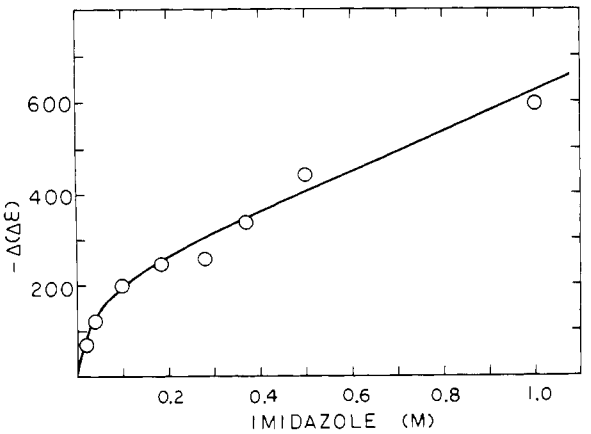

FIGURE 3: Peak-to-trough difference ($\Delta\epsilon_{289} - \Delta\epsilon_{295}$) produced by imidazole (see Figure 2) at pH 7.3. Solid curve is a theoretical fit discussed in the text.

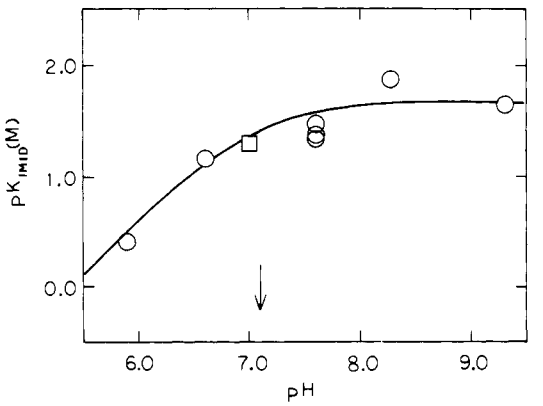

FIGURE 4: The pH dependence of the imidazole dissociation constant determined by the effect of imidazole on the K_i of iodide. Solid curve is a least-squares fit assuming dependence on a single ionizing group (arrow shows pK_a). Open square is a kinetically determined value previously obtained by direct inhibition of CO_2 hydration activity of HCAB. See text for details.

imidazole concentration. The slope of a plot of $\Delta\epsilon_{292}$ vs. imidazole concentration was -240 cm^{-1} M^{-1} per mol of imidazole in the case of tryptophan, while a similar plot for tyrosine at 286 nm had a slope of only -62 cm^{-1} M^{-1} per mol of imidazole.

Measurement of K_i for Iodide Binding in Presence and Absence of Imidazole. The dependence of $\Delta\epsilon_{299}$ on iodide concentration followed the behavior expected of a simple binding isotherm for the equilibrium:

$$E + I \rightleftharpoons EI \qquad K_i = [E][I]/[EI]$$

where E represents the enzyme and I the iodide inhibitor. The fraction of enzyme inhibited at any iodide concentration is defined as f_i and is then given by:

$$f_i \equiv \frac{[EI]}{[E] + [EI]} = \frac{\Delta\epsilon_i}{\Delta\epsilon_{max}}$$

where $\Delta\epsilon_i$ is the magnitude of $\Delta\epsilon_{299}$ for any concentration of iodide, [I], and $\Delta\epsilon_{max}$ is the value of $\Delta\epsilon_i$ when all the active sites are saturated with iodide. In most cases the iodide was in large excess over enzyme, so that [I] is equal to $[I]_0$. When this was not the case, corrections were made based on provisional estimates of $\Delta\epsilon_{max}$ that permitted the calculation of the fraction of enzyme binding I. The two unknowns, $\Delta\epsilon_{max}$ and K_i, were obtained by combining the above relations to a form permitting linearized plots:

$$(\Delta\epsilon_i)^{-1} = (K_i/\Delta\epsilon_{max})[I]^{-1} + (\Delta\epsilon_{max})^{-1}$$

This form is analogous to the Lineweaver–Burk plot of the Michaelis–Menten equation of enzyme kinetics and yields reliable results if properly weighted (Cornish-Bowden, 1976). We consequently obtained the parameters by a weighted least-squares procedure that assumed constant absolute errors in the $\Delta\epsilon_i$. The resulting values of K_i and $\Delta\epsilon_{max}$ are listed in Table II. It can be seen that, in all cases, the presence of imidazole decreases the affinity of HCAB toward iodide.

Calculation of the Imidazole Dissociation Constant for Binding to HCAB. The iodide binding data in the absence and presence of imidazole were analyzed on the assumption of competition between these two inhibitors for binding to the active site. The apparent K_i for iodide binding is then assumed to be influenced by imidazole binding at any pH according to the following relation:

$$K_{i,app} = K_i(1 + [Imid]/K_{imid})$$

where K_{imid} is the dissociation constant for imidazole, and K_i is the dissociation constant for iodide in the absence of imidazole. Solving for K_{imid} by this procedure yielded the values listed in Table II and plotted in Figure 4.

TABLE II: Dissociation Constants for Iodide and Imidazole Binding to HCAB from Difference Spectral Changes at 299 nm.[a]

pH	[Imidazole (M)]	K_i[b] (mM)	K_{imid}[c] (mM)
5.9		0.18	
5.9	0.50	0.43	380
6.6		0.27	
6.6	0.10	0.66	68
7.5		0.90	
7.5	0.05	1.95	42
7.5	0.10	3.6	33
7.5	0.30	7.1	44
8.3		2.0	
8.3	0.10	17.6	13
9.3		25	
9.3	0.10	133	23

[a] Measurements were at 25 °C in Bistris or Tris sulfate buffers of ionic strength 0.5. [b] Obtained by a weighted linear least-squares analysis of plots of $(\Delta\epsilon_i)^{-1}$ vs. $[\text{iodide}]^{-1}$. When imidazole was present, these represent the apparent K_i in the presence of the constant amount of imidazole shown in column 2. [c] Calculated from the effect of imidazole on the K_i assuming competitive binding of the two (see text).

Discussion

With the exception of imidazole, all the inhibitors of carbonic anhydrase studied here and elsewhere (King and Burgen, 1970) yield a characteristic difference spectrum in the aromatic region (cf. Figure 1), regardless of the structure of the inhibitor. In contrast, low imidazole concentrations generate initially a much smaller difference spectrum with a shifted peak at 294–295 nm. Higher imidazole concentrations produce an opposite sense difference spectrum characteristic of solvent perturbation (cf. Donovan, 1969) of exposed aromatic chromophores of the protein.

We have attempted to quantitatively account for the difference spectrum of imidazole in terms of these two effects. Figure 3 shows the experimentally determined ($\Delta\epsilon_{289} - \Delta\epsilon_{295}$) $\equiv \Delta(\Delta\epsilon)$ and a theoretical fit in which the $\Delta(\Delta\epsilon)$ was assumed to be given by the sum:

$$\Delta(\Delta\epsilon) = \left(\frac{[Imid]/K_{imid}}{1 + [Imid]/K_{imid}}\right)\Delta\epsilon_{max} + \left(\frac{\Delta\epsilon_{sp}}{[Imid]}\right)[Imid]$$

The first term represents the contribution of the difference spectrum produced by binding at the active site, while the second represents the solvent perturbation effect on the exposed chromophores of the protein. Since the K_{imid} was known at this pH, we adjusted the two parameters $\Delta\epsilon_{max}$ (= -200 cm^{-1} M^{-1}) and $\Delta\epsilon_{sp}$/[Imid] (= -430 cm^{-1} M^{-1} per mol of imidazole) to give the fit shown by the curve in Figure 3. This solvent perturbation parameter corresponds to a degree of exposure, R, of protein chromophores of 0.30 as follows. We neglect the effect of imidazole on the tyrosines in comparison to its effect on the tryptophans at the tryptophan difference maximum (cf. results of Lindskog and Nilsson, 1973), and we note that there are six Trp residues in HCAB (Andersson et al., 1972). Since $\Delta\epsilon_{sp}$ per mol of Trp per mol of imidazole was found to be -240, we find R to be:

$$R = -430/(6 \times 240) = 0.30$$

This degree of exposure compares very favorably with the results of Lindskog and Nilsson (1973) who found an average R of 0.35 for a variety of solvent perturbants of HCAB.

We conclude from the above analysis that imidazole does indeed produce a quite different inhibitor difference spectrum in HCAB, not only in the wavelength maximum (294–295 nm vs. 298–299 nm for the others), but also in its magnitude ($\Delta\epsilon_{295}$ = 200 cm^{-1} M^{-1} vs. 900–1200 cm^{-1} M^{-1}). This small magnitude and the complications from the solvent perturbation effect made it impractical to *directly* determine the K_{imid} for binding to the active site. We consequently attempted to measure K_{imid} indirectly by the effect of imidazole on the affinity of the enzyme for the inhibitor iodide. It was necessary to first establish that reliable values for the affinity of iodide could be obtained by difference spectroscopy. In this respect, iodide was chosen because it has been thoroughly studied (Whitney and Brandt, 1976; Khalifah, 1977) and extensive data were available on its binding over a wide range of pH. The pK_i values we obtained were in very good agreement with those reported by Whitney and Brandt (1976), the latter being measured by inhibition of the esterase activity of the enzyme at a somewhat lower ionic strength.

The calculated values of K_{imid} based on the assumption of competition with iodide are given in Table II and are plotted in the form of pK_{imid} vs. pH in Figure 4. Justification of the assumption of competition was based on the following considerations. The K_{imid} determined at pH 7.0 in a previous study of the inhibition of the CO_2 hydration activity (Khalifah, 1971), shown by the open square in Figure 4, was in excellent agreement with our determined values in this study. More importantly, the K_{imid} value determined through the iodide effect was independent of imidazole concentration (see pH 7.5 entries of Table II), as expected of strict competition. We conclude that within the accuracy of our data, iodide and imidazole bind competitively.

The pH dependence of the binding of imidazole to HCAB seen in Figure 4 is nearly unique[3] to the known behavior of the inhibitors of this enzyme (cf. Lindskog et al., 1971). In nearly all the known cases, the "catalytically essential" ionization of the active site is believed to control the binding of inhibitors (Lindskog, 1966), so that *formally* the acid form of the enzyme active site appears to bind the basic (anionic) form of the inhibitors when the latter can ionize. Thus, in the case of halide binding, the affinity is maximal at low pH and is abolished at high pH, while with sulfonamides a bell-shaped profile is obtained (Lindskog et al., 1971). In contrast, the binding of imidazole seen in our study appears maximal at *high* pH and is abolished at low pH. The solid curve of Figure 4 is a least-squares fit to a situation where the K_{imid} depends on a *single* ionization:

$$K_{imid} = K^0_{imid}(1 + [H^+]/K_a)$$

where K^0_{imid} is the high pH limit of K_{imid} and K_a is the ionization constant governing the binding. The least-squares fit of the data yielded a K^0_{imid} of 0.022 M and a pK_a of 7.10. This latter value is much closer to that for the ionization of imidazole at similar ionic strengths (Khalifah, 1971) than to that of the catalytically essential active-site group which is at least 7.6 (Khalifah, 1971). Thus, within the limits of our data, we can conclude that the neutral form of imidazole appears to inhibit the enzyme regardless of the ionization state of the enzyme.[4]

The above results all point to a unique mode of binding of imidazole to the active site. The implications of this to the binding of the substrate CO_2 may be important, since imidazole is the only known competitive inhibitor of CO_2 in this enzyme (Khalifah, 1971). It is fortunate that the crystal structure of the imidazole complex of HCAB has recently become available (Kannan et al., 1977), so that it is possible to ask whether the results we obtained can be rationalized in terms of the three-dimensional model. Kannan and co-workers have, indeed, found an unusual binding mode for imidazole. Rather than displacing the solvent (fourth) ligand of the distorted-tetrahedral zinc, imidazole binds in a hydrophobic pocket near the metal at a distance of 2.7 Å and may be considered as a weak fifth ligand of the metal (Kannan et al., 1977). These results have support from ^{1}H NMR studies (Campbell et al., 1974).

The above mode of coordination is fully consistent with all our observations. The linkage between *anion* binding and active-site ionization presumably arises because the latter is probably the ionization of the aquo ligand of the zinc (Khalifah et al., 1977), so that anions compete with OH^- for binding to the fourth ligand position of the zinc (Khalifah, 1977). Imidazole, by not displacing the solvent ligand, becomes independent of this zinc aquo ligand ionization. The sole ionization governing the binding is probably that of imidazole. This is expected based on the nature of interaction of this ligand with transition metals (Sundberg and Martin, 1974; Martin, 1974). It is interesting also that the above mode of binding of imidazole may provide an explanation for the suppressed and shifted difference spectrum produced by imidazole. The binding of other inhibitors such as iodide at the "anion pocket" adjacent to the zinc, at least in HCAC, involves contacts with the side chain of Thr-199 and its NH group, as well as van der Waals contacts with Leu-198 *and* the ring of the buried Trp-209 (Vaara, 1974). The different spectrum of imidazole is then presumably a reflection of its greater distance from Trp-209, although this is not entirely clear from the description of Kannan et al. (1977). The differing inhibitor difference

[3] The *reversible* inhibition of HCAB by iodoacetamide shows a qualitatively similar pH dependence, but some complications arise in the measurement of its binding constant (Whitney, 1970). It is interesting that a third "neutral" inhibitor, aniline, has a pH dependence of its binding similar to the anions in that the affinity is maximal at *low* pH (Appleton and Sarkar, 1975).

[4] No *active-site* histidine has been found to have a pK_a close to 7.1 (Campbell et al., 1974). Another ionizing group in the active site is the buried Glu-106 of unknown pK whose ionization may be intimately coupled to that of the aquo ligand of the zinc (Kannan et al., 1977). Note that the assumption that only an active-site group controls imidazole binding requires, for consistency with the observed pH dependence, that both ionization states of imidazole (protonated and unprotonated) bind equally well. The resulting coordination of a protonated imidazole to the metal is highly implausible (Sundberg and Martin, 1974).

spectra of imidazole and the other anions thus may be a reflection of the differences in their orientation to Trp-209 and not just a difference in property such as charge, since, for example, the neutral ethyl carbamate inhibitor behaves like the anions (cf. Table I).

Finally, it is pertinent to note that a proposal has been advanced for the binding mode of the CO_2 substrate by analogy with imidazole (Kannan et al., 1977). Such similarity is consistent with the difference spectra and the pH dependence found in this study, the latter since the CO_2 hydration kinetics (Khalifah, 1971) have not yet shown any dependence of CO_2 binding on active-site ionizations (Khalifah and Edsall, 1972). However, a discrepancy does arise when considering the relation of CO_2 and imidazole binding to binding of anionic inhibitors. The inhibition by anions has been found to be *non-competitive* with respect to CO_2 in kinetics studies of the enzyme activity[5] (Kernohan, 1965). On the other hand, the binding of iodide and imidazole seen in our study is fully consistent with competition of these two inhibitors. The difference may be related to the larger size of imidazole compared to CO_2. However, it should be kept in mind that the kinetic criterion of competitive binding need not result from steric overlap of the competing ligands. Further attempts to locate the CO_2 binding site, as for example by NMR methods (Stein et al., 1977), should be actively pursued.

Acknowledgments

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[5] A *nonkinetic* study of CO_2 binding concluded that anions displace the bound CO_2 observed by infrared difference spectroscopy (Riepe and Wang, 1968). For reasons discussed elsewhere (Khalifah, 1971), the bound CO_2 observed in that study is highly unlikely to be the *substrate* CO_2, since it characteristics are incompatible with kinetic studies of the catalytic activity of the enzyme.