Tanabe, A., Nielsen, T. B., Sheela Rani, C. S., & Field, J. B. (1985) *Arch. Biochem. Biophys. 243*, 92–99.
Thomas, J. A., Schlender, K. K., & Larner, J. (1968) *Anal. Biochem. 25*, 486–499.
Uchida, T., & Filburn, C. R. (1984) *J. Biol. Chem. 259*, 12311–12314.
Vallee, B. L., & Hoch, F. L. (1955) *Proc. Natl. Acad. Sci. U.S.A. 41*, 327.
Wise, B. C., Raynor, R. L., & Kuo, J. F. (1982) *J. Biol. Chem. 257*, 8481–8488.
Wooten, M. W., & Wrenn, R. W. (1985) *Cancer Res. 45*, 3912–3917.

Interaction of the Unique Competitive Inhibitor Imidazole and Related Compounds with the Active Site Metal of Carbonic Anhydrase: Linkage between pH Effects on the Inhibitor Binding Affinity and pH Effects on the Visible Spectra of Inhibitor Complexes with the Cobalt-Substituted Enzyme†

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ABSTRACT: Previous studies on the interaction of carbonic anhydrase (CA) with the unique CO_2 competitive inhibitor imidazole and related compounds were all interpreted as showing that an ionizable water ligand on the metal of this zinc metalloenzyme is *not* displaced by inhibitor binding. Internal inconsistencies in the pH dependence of binding and the pH dependence of the visible spectra of complexes with cobalt-substituted enzyme prompted us to reinvestigate this binding. Visible spectroscopy was used to measure the binding of imidazole and 1,2,4-triazole to Co(II)-substituted human CA I and active site carboxymethylated human CA I (CmCA I) and the binding of 1,2,4-triazole to bovine Co^{II}CA II. The limiting visible spectra for these enzyme–inhibitor adducts were also computed and examined for pH dependence. It was shown that the pK_a of visible spectral changes can be independently predicted from studies on the pH dependence of binding. After consideration of possible contributions from effects of His-200 ionization in CA I and CmCA I, and His-64 in CA II, the pH effects on binding affinity and spectra were found to be of the correct magnitude to establish linkage between binding and an ionization. It was also shown, however, that pH effects on binding and spectra cannot distinguish whether neutral imidazole binds to both ionization forms of the enzyme (Zn-OH_2 and Zn-OH) or whether neutral imidazole and its anion both bind to only the acid form of the enzyme, presumably after displacing the water. These findings have implications to the crystallographic interpretations on the imidazole–enzyme complex and to the catalytic mechanism of CO_2 hydration.

It was discovered some 15 years ago that imidazole is a carbonic anhydrase (EC 4.2.1.1) isozyme I inhibitor that is uniquely competitive with the physiological substrate carbon dioxide (Khalifah, 1971). To date, only one other inhibitor has been demonstrated to be similarly competitive with carbon dioxide, this being phenol in its inhibition of the other major isozyme II (Simonsson et al., 1982). The understanding of the mode of binding of imidazole to CA I[1] is thus of special mechanistic significance (Kannan et al., 1977; Silverman & Vincent, 1983). This is made even more so by the difficulty of studying an isolated enzyme–carbon dioxide complex (Riepe & Wang, 1968; Khalifah, 1971; Williams & Henkens, 1985; Stein et al., 1977) and by the absence of true homologues of this small substrate.

Besides being competitive with CO_2 and being an uncommon neutral inhibitor, imidazole differs from most other inhibitors of carbonic anhydrase in three important respects. First, we note that the overwhelming majority of anionic inhibitors [cf. reviews by Maren (1967, 1976)], including the ionizable sulfonamides (Kanamori & Roberts, 1983; Blackburn et al., 1985), are known to bind by displacing the ionizable water ligand in the coordination sphere of the active site zinc (Lindskog, 1982, 1983; Lindskog et al., 1971; Bertini & Luchinat, 1983). However, when Kannan and co-workers carried out crystallographic studies of the imidazole–CA I complex, they found that imidazole does not displace the catalytically essential water ligand of the metal (Kannan et al., 1977; Kannan, 1980). Instead, it appears to bind as a

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[1] Abbreviations: CA I and II, carbonic anhydrase isozymes I and II (formerly referred to as B and C type); CmCA I, CA I carboxymethylated at its active site His-200; Bis-Tris, [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane; MES, 4-morpholineethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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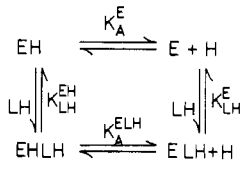
Scheme I



$$K_A^{ELH}/K_A^E = K_{LH}^{EH}/K_{LH}^E$$

distant and distorted fifth "ligand" of the metal with an unusually long ligand–metal distance of 2.7–2.8 Å. Second, it was discovered, again in contrast to the binding of virtually all inhibitors of this enzyme, that the affinity of neutral[2] imidazole for CA I was independent of pH (Wolpert et al., 1977; Bauer et al., 1977; Alberti et al., 1981). In view of the crystallographic findings on the complex of imidazole with CA I, it was suggested that the pH independence of imidazole binding was due to the lack of competition with solvent hydroxyls upon binding (Wolpert et al., 1977; Bauer et al., 1977). The catalytic consequences of the implied pentacoordination and the lack of displacement of the crucial solvent ligand were quickly appreciated, and catalytic mechanisms were advanced that were based on carbon dioxide mimicking the proposed binding of imidazole (Kannan et al., 1977; Kannan, 1980; Bauer et al., 1977; Lindskog et al., 1984).

A third unusual feature of imidazole binding was the discovery (Bauer et al., 1977) that the visible spectrum of its *complex* with Co(II)-substituted CA I underwent large pH-dependent intensification at alkaline pH. The midpoint of this spectral transition appeared to be close to pH 8. Since the ionization of the coordinated water in the *uninhibited* enzyme occurs with a pK near 7.5, the spectral transition in the complex with imidazole was attributed (Bauer et al., 1977) to perturbation by the ionization of an adjacent, nondisplaced water ligand. Alberti et al. (1981) later enlarged on the imidazole results and interpretations and extended the study to include the stronger inhibition of human CA I and bovine CA II by the imidazole analogues tetrazole, 1,2,3-triazole, and 1,2,4-triazole. The spectra of the complexes of the latter compounds with the cobalt enzymes were reported to be pH independent, in contrast to the complex pH dependence of their binding. Kinetic studies on these inhibitors have recently been reported (Tibell et al., 1985).

We report here that the available data summarized above on the pH dependence of the affinity and spectral properties appear to be inconsistent with the interpretations advanced regarding the presence of an ionizable water ligand in the complexes. Since the pK_a seen for the spectral change implies an increase in the pK_a of the coordinated water ionization from its value in the uninhibited enzyme, a reciprocal effect of pH on binding affinity should have been observed. It can be readily shown that, *if ligand binding perturbs an ionization on the enzyme or on the ligand, then proton binding will perturb the affinity of the ligand for the enzyme.* Scheme I indicates how ligand binding and an assumed enzyme ionization can be linked functions (Wyman, 1964) with reciprocal effects expressed by eq 1. Here the designations E and EH

$$pK_a^{ELH} - pK_a^E = pK_{LH}^{EH} - pK_{LH}^E \quad (1)$$

refer to free enzyme with zinc-bound hydroxyl or water, respectively, and LH represents neutral imidazole. The implied absence of such a linkage in the reported data and the use of Co(II)-substituted enzyme for spectral studies while the Zn(II) form was used for affinity measurements both prompted us to reinvestigate binding and spectral changes. We now do indeed find that the new data are compatible with a linkage between the ionization affecting the spectral changes and inhibitor binding. Just as important, we show that the linked ionization can formally belong either to the enzyme, e.g., that of a coordinated water ligand, or to the inhibitor, namely, the deprotonation of the "pyrrole" nitrogen in imidazole and its analogues (Sundberg & Martin, 1974). The latter possibility, in which imidazole coordinates to the metal at high pH as the anion, was first mentioned by Bertini and Luchinat (1983) and has ample precedent in small metal complexes (Sigel & Martin, 1982), though not proteins [cf. Valentine and Pantoliano (1983)].

Experimental Procedures

Enzymes and Chemicals. Human carbonic anhydrase isozyme I was prepared from freshly outdated erythrocytes according to the affinity chromatography method developed earlier (Khalifah et al., 1977). Bovine carbonic anhydrase II was purchased from Sigma. Replacement of the intrinsic Zn(II) with Co(II) was carried out by first removing the Zn(II) according to the pyridine-2,6-dicarboxylate procedure (Hunt et al., 1977), followed by stoichiometric addition of Specpure-grade cobalt sulfate (Johnson-Matthey). Cobalt-substituted CA I carboxymethylated specifically at His-200 [Co^{II}CmCA I] was prepared by reacting Co^{II}CA I with 15 mM bromoacetate in 0.1 M Bis-Tris buffer at pH 6.8 in the dark at room temperature for 6–7 h, followed by extensive dialysis. Protein concentrations were determined from the absorbance at 280 nm (Lindskog et al., 1971) or from the visible spectrum in the case of the Co(II)-substituted enzymes, published results being used (Whitney, 1970; Taylor et al., 1970). Imidazole was grade III from Sigma and was used without further purification. 1,2,4-Triazole was obtained from Aldrich.

Spectrophotometry. All spectra were recorded on a Varian Cary 210 spectrophotometer equipped with a digital interface port and interfaced to an Apple II+/IIe via a parallel interface card (SSM AIO). Software for scanning, storage, and manipulation of spectra was developed in this laboratory and was written in Microsoft TASC-compiled BASIC language.

Inhibitor Binding Measurements. Inhibitor dissociation constants for imidazole and 1,2,4-triazole binding to the Co(II)-substituted enzymes were determined by visible spectral titrations at ambient temperature (25 °C). Complete spectra were recorded for each addition of imidazole or triazole, and the absorbance changes at a wavelength of maximal difference between native and inhibited enzyme were analyzed as a function of inhibitor concentration by nonlinear least-squares procedures. Corrections for light scattering and dilution were routinely carried out. Imidazole concentrations were maintained below about 0.3 M in order to prevent possible denaturation of the enzyme. Generally, 0.05 M buffers (MES, HEPES, Tris, and β-alanine) of ionic strength 0.2 (K_2SO_4) were used.

Computation of Limiting Spectra of Enzyme–Inhibitor Complexes. In order to obtain the spectra of fully inhibited enzyme at each pH, an extrapolation procedure was necessary, since the highest inhibitor concentration used (0.3 M) typically produced 80–90% saturation. The following procedure was thus devised and implemented. With the K_i determined at each pH from absorbance changes at wavelengths of maximal

[2] It has been shown (Wolpert et al., 1977) that protonation of imidazole prevents its binding to CA I. All K_i values reported in the present work have been computed on the basis of the concentration of the neutral species.

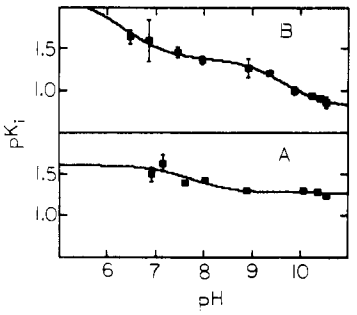


FIGURE 1: Dependence of the imidazole inhibitor dissociation constant of $Co^{II}CA$ I (A) and $Co^{II}CmCA$ I (B) on pH. The K_i values were obtained from visible spectral titrations. The curve of (A) represents a weighted nonlinear regression fit to eq 3 of the text, with parameters given in Table I. In (B), the curve represents a fit to a more complex function in which an additional ionization at low pH was included, as discussed in footnote 3 and the text. The ionic strength was maintained at a minimum of 0.2 with K_2SO_4.

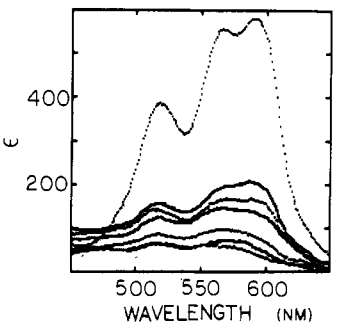


FIGURE 2: Visible absorption spectra for the imidazole complexes of $Co^{II}CmCA$ I (solid curves) and $Co^{II}CA$ I (broken curve). Spectra were computed from the same spectral titrations used in Figure 1 by the procedure described under Experimental Procedures. All samples were at ionic strength 0.2 established with K_2SO_4. pH values in the order of decreasing absorbance were as follows: 10.56 (broken curve), 10.56, 10.26, 9.90, 9.38, 8.95, and 7.98.

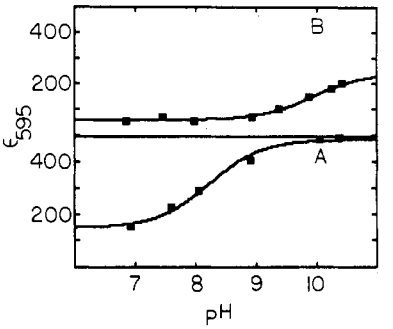


FIGURE 3: pH dependence of the 595-nm molar extinction coefficient of the limiting spectra of the imidazole complexes with $Co^{II}CA$ I (A) and $Co^{II}CmCA$ I (B). The solid curves represent three-parameter nonlinear regression fits of the experimental data (Table I).

difference that presumably had the greatest accuracy, a constrained least-squares analysis with K_i fixed was performed at each other wavelength in order to determine the extrapolated molar extinctions for the complexed enzyme. The procedure was programmed for computation on the Apple II+/IIe.

RESULTS

pH Dependence of Visible Spectra of $Co^{II}CA$ I and Its Imidazole Complex. Visible spectral changes accompanying the binding of imidazole to $Co^{II}CA$ I have been extensively described by Bauer et al. (1977) and Alberti et al. (1981). We report only the following spectral pK_a results that are necessary for our analysis, obtained under our specified values of buffer composition, temperature, and ionic strength. We found that the principal visible spectral transition of the uninhibited $Co^{II}CA$ I, presumably reflecting the water ligand ionization, occurs at 7.5 ± 0.2. Under similar buffer and ionic strength conditions, we found the pK_a governing the spectral intensification of the *limiting* imidazole–$Co^{II}CA$ I complex to occur at 8.2 ± 0.2 (Figure 3A). Bauer et al. (1977) reported an apparent pK_a of 7.9 for the spectral changes of $Co^{II}CA$ I in the presence of constant 0.33 M imidazole. A pK_a close to 8.0 can be discerned in the limiting imidazole–$Co^{II}CA$ I spectra of Alberti et al. (1981), obtained under unknown buffer and ionic strength conditions.

pH Dependence of Imidazole Binding to $Co^{II}CA$ I. The K_i for the imidazole inhibition of $Co^{II}CA$ I was determined by monitoring the visible spectral changes induced by imidazole addition. Figure 1A shows the pK_i values obtained over the pH range of 7–10. The measurements could not be extended to lower pH due to the vanishingly small difference between the spectra of the enzyme and its complex. A small but significant increase in binding affinity is observed at low pH, with a pK_i amplitude of 0.33. The K_i values were computed on the basis of the concentration of neutral imidazole, corrections being made by use of an assumed pK_a of 7.14 for protonation of imidazole at this ionic strength [cf. Khalifah (1971)]. It has been shown (Wolpert et al., 1977) that protonation of imidazole prevents its binding to the enzyme.

Binding of Imidazole to Human $Co^{II}CmCA$ I. Similar measurements were carried out on the carboxymethylated derivative of CA I. This form is known to have intrinsic residual activity and inhibitor binding capacity, but the ionization of the water ligand of the metal is shifted from 7.5 to 9.2 (Whitney, 1970; Taylor et al., 1970). Figure 1B shows the pK_i values obtained for inhibition of $Co^{II}CmCA$ I by imidazole over the pH range of 6.5–10.5. The pK_i for the modified enzyme shows a greater dependence on pH. An inflection of amplitude 0.57 is seen in the pH range above 9, with an additional increase in affinity being apparent below pH 7. The curve drawn through the data will be discussed below.

Visible Spectra of Complexes of Imidazole with $Co^{II}CmCA$ I. The limiting spectra of the adducts of imidazole with $Co^{II}CmCA$ I have been computed and are shown in Figure 2. The imidazole–$Co^{II}CmCA$ I complex appears to have a generally similar spectrum to that of the complex with $Co^{II}CA$ I at neutral pH, but it differs markedly in the alkaline range. Only a modest, though quantifiable, intensification is seen near 590 nm, with the molar extinction remaining below 250, compared to the value near 600 seen with $Co^{II}CA$ I (Bauer et al., 1977; Alberti et al., 1981). Figure 3B shows a plot of the pH dependence of this change, whose pK_a is found to be 10.0 ± 0.2 by nonlinear least-squares analysis. The spectra of *uninhibited* $Co^{II}CmCA$ I are very similar to those of uninhibited $Co^{II}CA$ I, except that the pK_a of the spectral change that is attributable to the ionization of the water ligand of the metal occurs at pH 9.2 ± 0.2 in the modified enzyme. This is in full agreement with earlier studies (Whitney, 1970; Taylor et al., 1970).

Binding of 1,2,4-Triazole to $Co^{II}CA$ I. Similar studies were carried out on the inhibition and spectral properties of complexes of this imidazole analogue with human $Co^{II}CA$ I. Figure 4B shows that the limiting spectrum of the complex with this enzyme is fairly pH independent, but it does show a previously unreported small decrease in intensity below pH 6. If we assume that at this wavelength the low-pH extinction coefficient is similar to that of the enzyme–imidazole complex, i.e., near 100, we estimate by a nonlinear least-squares fit that an ionization of $pK = 5.1 \pm 0.1$ governs this change. The pH dependence of the dissociation constant for binding (Figure

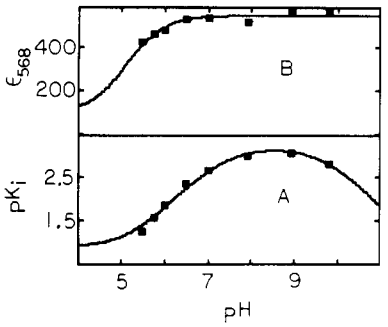


FIGURE 4: (A) pH dependence of the 1,2,4-triazole inhibitor dissociation constant of $Co^{II}CA$ I. The K_i values were obtained from visible spectral titrations. The curve represents a weighted nonlinear regression fit to eq 3 of the text, with parameters given in Table I. (B) pH dependence of the 568-nm extinction coefficient of the limiting 1,2,4-triazole complex with $Co^{II}CA$ I. The solid curve represents a three-parameter nonlinear regression fit of the experimental data to a single ionization function (Table I).

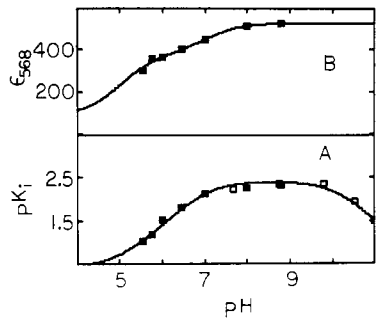


FIGURE 5: (A) pH dependence of the 1,2,4-triazole inhibitor dissociation constant of bovine $Co^{II}CA$ II. The K_i values were obtained from visible spectral titrations. The open squares are data taken from Alberti et al. (1981). The curve represents a weighted nonlinear regression fit to eq 3 with all the data (Table I). (B) pH dependence of the 568-nm extinction coefficient of the limiting 1,2,4-triazole complex with bovine $Co^{II}CA$ II. The solid curve represents a four-parameter nonlinear regression fit of the experimental data to a two-pK_a function in which the lower pK_a was constrained to 5.0.

4A) is much more complex than that of imidazole, and its analysis will be discussed further below.

Binding of 1,2,4-Triazole to Bovine $Co^{II}CA$ II. Similar studies were carried out on the binding of this inhibitor to the high specific activity isozyme. The dependence of the inhibition constant on pH is shown in Figure 5A, along with results (open squares) from Alberti et al. (1981) that show the decreased binding at high pH. Our data are in good agreement also with the findings of Tibell et al. (1985) on the triazole inhibition of the esterase activity of the closely similar human CA II. The overall bell-shaped dependence is similar to that seen with $Co^{II}CA$ I. However, the spectrum of the inhibitor–enzyme adduct has more complex pH dependence (Figure 5B) than seen with CA I, with a possible dependence on two ionizations. One ionization appears to have a pK_a of 7.0 ± 0.2, while the decrease at lower pH can be roughly estimated to be governed by a pK_a = 5.0 ionization, if the limiting low-pH extinction coefficient is assumed to be in the neighborhood of 100. The curve drawn through the data represents a nonlinear least-squares fit where one ionization was constrained to be 5.0. It was unfortunately not feasible to extend the data to the lower pH range needed to ascertain this.

Discussion

Earlier studies on the inhibition of $Zn^{II}CA$ I by imidazole have revealed no dependence of the binding on pH. The most extensive studies in this regard (Wolpert et al., 1977) utilized an indirect method for measuring binding, using competition with iodide binding (Khalifah, 1980) and observation of iodide-induced UV difference spectral changes. In the present work on the $Co^{II}CA$ I and $Co^{II}CmCA$ I, the binding was directly studied through the changes in the cobalt visible spectra induced by imidazole. We now do find a small but quantifiable pH dependence of the inhibition dissociation constant for both $Co^{II}CA$ I and $Co^{II}CmCA$ I in the vicinity of the pH where the water ligand is assumed to ionize. A more quantitative linking of the pH dependence of binding to the pH dependence of spectral changes requires reference to specific models of binding, particularly if the complex behavior of 1,2,4-triazole is to be taken into account.

Scheme I accomodates the situation where imidazole binds without displacing a solvent ligand of the metal. Neutral imidazole (LH) is assumed to bind to both ionization forms (zinc–water and zinc–hydroxide) of the enzyme. In order to account for the decreased binding of imidazole analogues at alkaline pH, Alberti et al. (1981) proposed that deprotonation of the "pyrrole" nitrogen of these compounds abolishes the binding to the enzyme. This ionization occurs with a pK_a above 14 in imidazole (Sundberg & Martin, 1974), but it is much more acidic in the triazoles (pK_a values of 9.4 and 10.3 for 1,2,3- and 1,2,4-triazoles) and tetrazole (pK_a of 4.9) (Alberti et al., 1981). We add this feature to the model of Scheme I by explicitly including the ligand ionization equilibrium of eq 2. Here L denotes the anion in which the "pyrrole" nitrogen is deprotonated. We shall refer to it as the WBA model, since it incorporates the concepts of Wolpert et al. (1977), Bauer et al. (1977), and Alberti et al. (1981) in that (1) imidazole binds to both ionization forms of the active site, presumably not displacing the ionizable water ligand of the metal, and (2) the imidazole anion (and cation) has no affinity for the enzyme.

$$LH = L + H \qquad K_a^{LH} = [L][H]/[LH] \qquad (2)$$

The WBA model leads to the following expression for the dependence of the apparent pK_i on pH:

$$K_i = \frac{(K_a^L K_{LH}^{EH}/K_a^{ELH})(1 + [H]/K_a^E)(1 + [H]/K_a^L)}{([H]/K_a^E)(1 + [H]/K_a^{ELH})} \qquad (3)$$

The cyclic nature of the equilibria of Scheme I permits the expressing of any of the four equilibrium constants in terms of the other three. We have chosen to include the equilibrium constant K_a^{ELH}. Within the framework of the WBA model, this constant *formally* represents the ionization of an enzyme group within the enzyme–inhibitor complex, such as the coordinated water ligand that was proposed to be not displaced by binding. It can also be viewed as the pK_a for a change in the structure of the enzyme–inhibitor complex that may be accompanied by spectral alterations. In this sense, we conclude from inspection of eq 3 that *it is possible, in principle, to predict the pK_a of the ionization affecting the spectra of the inhibitor–enzyme complexes from completely independent measurements of the pH dependence of the apparent pK_i.*

The pH dependence of the apparent pK_i of eq 3 is fully capable of describing the inhibition by imidazole as well as by triazole. This is qualitatively illustrated in Figure 6 for sample calculations where pK_a^E is taken as 7, pK_a^L is taken as 12, and pK_a^{ELH} is varied. The pH dependence for this typical case is seen to be a sensitive function of the *difference* between pK_a^E and pK_a^{ELH}. Near coincidence of these values implies little perturbation of the enzyme ionization by ligand binding and correspondingly little pH dependence of the apparent pK_i in the range of these ionizations. This is the case apparently seen

Table I: Summary of Ionization and Inhibitor Dissociation Constants Obtained for Inhibition of Co(II)-Substituted Carbonic Anhydrases by Imidazole and 1,2,4-Triazole[a]

observed	enzyme	inhibitor	pK_a^E	pK_a^L	pK_a^{ELH}	pK_{LH}^{EH}	pK_L^{EH}
ϵ_{595}	Co^{II}CA I	imidazole			8.2 ± 0.2		
$K_{i,app}$	Co^{II}CA I	imidazole	7.6 ± 0.5	14.5	7.9 ± 0.4	1.6 ± 0.1	(8.2)
ϵ_{640}	Co^{II}CA I	none	7.5 ± 0.2				
$K_{i,app}$	Co^{II}CA I	triazole	7.4 ± 0.2	9.7 ± 0.3	5.1	0.89 ± 0.3	(5.4)
ϵ_{568}	Co^{II}CA I	triazole			5.1 ± 0.1[b]		
ϵ_{595}	Co^{II}CmCA I	imidazole			9.9 ± 0.2		
$K_{i,app}$	Co^{II}CmCA I	imidazole	9.4 ± 0.2	14.5	10.0 ± 0.2	2.1 ± 0.1	(6.6)
ϵ_{640}	Co^{II}CmCA I	none	9.2 ± 0.2				
ϵ_{640}	Co^{II}CA II	none	6.8 ± 0.1				
$K_{i,app}$	Co^{II}CA II	triazole	7.0 ± 0.1	10.2 ± 0.1	5.0	0.41 ± 0.02	(5.6)
ϵ_{568}	Co^{II}CA II	triazole			5.0		
ϵ_{568}	Co^{II}CA II	triazole			7.0 ± 0.2[c]		
$K_{i,app}$	Co^{II}CmCA I	imidazole	6.0[d]		6.7 ± 0.2[d]	(1.4)[e]	

[a] All data were obtained at ambient temperature and at an ionic strength of 0.2 adjusted with K_2SO_4. Columns 4–6 respectively are ionization constants of free enzyme (zinc–water), free ligand ("pyrrole" nitrogen deprotonation), and enzyme–inhibitor complex (either ligand or enzyme ionization within complex, depending on model adopted). Column 7 gives inhibition constants (pK_i) in the acid limit representing intrinsic binding of the acid form of the enzyme to the neutral form of the inhibitor. Column 8 presents computed pK_i for intrinsic binding of anions of inhibitors to the acid form of the enzyme, with fitted or constrained constants in the preceding columns, in conjunction with the linkage relation given by eq 4 of the text (BL model). Fitted quantities are given with standard deviations, while entries without such deviations represent constrained values not varied in fits. [b] Value obtained by constraining the acid-limit species to an ϵ_{568} of 100. [c] Secondary ionization attributed to His-64 (see footnote 4 and the text). [d] Secondary ionization attributed to His-200 (see footnote 3 and the text). [e] This value pertains to the affinity of neutral imidazole to the acid form of the enzyme (zinc–water) with His-200 not protonated. Entry above (2.1) refers to neutral imidazole binding to enzyme when His-200 is protonated.

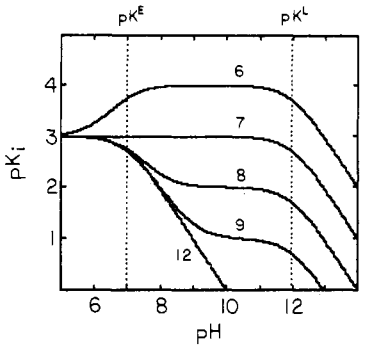


FIGURE 6: pH dependence of the apparent pK_i of eq 3 in the text on the pK_a of the inhibitor–enzyme complex (pK_a^{ELH}) for the case where the free enzyme pK_a^E is 7.0 and the free ligand pK_a^L is 12.0. In decreasing order at high pH, the value of pK_a^{ELH} was taken as 6.0, 7.0, 8.0, 9.0, and 12.0. Note the characteristic dependence of the curves on the *difference* between pK_a^{ELH} and pK_a^E. The equality of these parameters results in apparently pH-independent binding below pK_a^L.

with imidazole inhibition of CA I. On the other hand, inspection of the data on 1,2,4-triazole binding to CA I and II (Figures 4A and 5A) and reference to Figure 6 immediately suggest that the pK_a^{ELH} in these enzyme–inhibitor complexes must be significantly less than pK_a^E for the uninhibited enzyme.

The binding data for imidazole (Figure 1) and triazole (Figures 4A and 5A) have been fit by nonlinear least-squares to eq 3, and the results are summarized in Table I, which includes for comparison the fits for the pH variation of the visible spectra. In the case of imidazole binding, an inflection of amplitude 0.3 occurs with Co^{II}CA I in the pH range near 8, while a larger change (amplitude 0.57) occurs in Co^{II}CmCA I in the pH range near 10.[3] In both cases, the observed pH dependence implies, by eq 1, that binding of imidazole should lead to an increase in the linked enzyme ionization by the corresponding amplitudes. The observation of spectral perturbations (Table I) governed by pK_a values of 8.2 ± 0.2 and 10.0 ± 0.2 for imidazole complexes of Co^{II}CA I and Co^{II}CmCA I, respectively, is in good accord with these expectations, since the corresponding water ligand ionizations in the uninhibited enzyme are at 7.5 and 9.2 (Whitney, 1970; Taylor et al., 1970; Khalifah et al., 1977; Lindskog, 1982, 1983; Bertini & Luchinat, 1983).

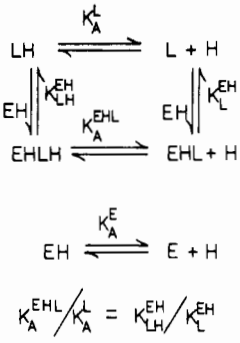
A similar analysis of the binding data for triazole inhibition of Co^{II}CA I and II confirms that the pK_a of the linked group must be *lower* than that of the enzyme pK_a^E by about 2 or more pH units. This analysis prompted us to search for low-pH effects on the spectra of the adducts of Co^{II}CA I and Co^{II}CA II with 1,2,4-triazole that previously had escaped attention (Alberti et al., 1981). The spectral data of Figures 4B and 5B are suggestive of the presence of low-pH perturbations, and a self-consistent fit to binding and spectral data could be obtained by assuming a pK_a of about 5.0–5.1 in each case. In the case of Co^{II}CA II (Figure 5B), a more prominent ionization having a pK_a of 7.0 ± 0.2 affects the spectrum that is not reflected in the binding. It is most likely that of active site His-64 [cf. Campbell et al. (1975)].[4] Tibell et al. (1985) have recently shown for bovine CA II that at much lower ionic strength the influence of this ionization on the binding can become quite prominent.

The above results and analyses can be construed as providing quantitative support for the linkage between binding of imidazole and analogues and the ionization of a nondisplaced water ligand, as described by the WBA model. However, they do not constitute a sufficient proof. The major difficulty arises from the fact that it has not been *independently* established that the ionizing group perturbing the spectra of the complexes is indeed that of a coordinated water ligand. This is in contrast, for example, to the direct ^{13}C NMR measurement of the

[3] The curve drawn through the data for Co(II)CmCA I (Figure 1B) incorporates the effect of an additional enzyme ionizing group (pK_{a1}^E), presumably His-200, to take into account the changes at low pH. The calculated pK_a of 6.7 ± 0.2 for this perturbing group in the complex (K_{a1}^{EL}) makes its assignment to His-200 reasonable. This side chain has a pK_a of 6.0 in the free enzyme (pK_{a1}^E) (Khalifah et al., 1977), and other inhibitors are known to increase its pK_a by about an order of magnitude (Khalifah, 1977; Khalifah & Morley, 1984). His-200 is much less sensitive to inhibitors in CA I (Campbell et al., 1974), and no such perturbation can be discerned in our limited data (Figure 1A) anyway.

[4] Campbell et al. (1975) have shown that the pK_a of the active site His-64 is 7.12 in uninhibited human CA II, but only a small increase (0.1–0.2) in this pK_a is seen upon binding of the inhibitors (Campbell et al., 1975; Tibell et al., 1984). The pK_a of the spectral change in the 1,2,4-triazole complex (Figure 5B) thus indicates that His-64 is insufficiently perturbed to warrant its inclusion in our quantitative analysis of the binding.

Scheme II



$$K_A^E \quad EH \rightleftharpoons E + H$$

$$K_A^{EHL} / K_A^L = K_{LH}^{EH} / K_L^{EH}$$

microscopic pK_a of His-200 in the presence and absence of inhibitors that permitted the unambiguous identification of its linkage to inhibitor binding (Khalifah, 1977).

Serious objections can also be raised regarding its physical validity. Foremost among these is that the visible spectra of the imidazole complex with CA I and 1,2,4-triazole complexes with CA I and II are sufficiently intense (molar extinction coefficients close to 600) to be unambiguously assigned as tetrahedral (Bertini & Luchinat, 1983, 1984; Bertini et al., 1984). Coordination of a solvent molecule along with imidazole or 1,2,4-triazole at high pH is incompatible with such tetrahedral coordination, given that the metal is firmly coordinated to three histidyl side chains from the protein (Lindskog, 1982, 1983; Lindskog et al., 1971). Similarly, the relaxation effects of the $Co^{II}CA$ on the solvent water protons are completely abolished by complexation with imidazole and its analogues at all pH values studied (Alberti et al., 1981). As pointed out by Alberti, this latter result does not prove the absence of a coordinated solvent molecule in these complexes. However, it clearly highlights the fact that *there is no direct evidence from any solution study for the presence of a simultaneously coordinated solvent molecule in these complexes.* It may also be significant that, contrary to expectations from the WBA model, the analogue with the lowest pK_a for "pyrrole" nitrogen deprotonation (tetrazole, $pK_a = 4.9$) actually exhibits the strongest affinity for CA below pH 7 (Alberti et al., 1981).

In view of the above, we have considered the possibility, first raised by Bertini and Luchinat (1983) but not elaborated further by them, that the origin of the pH dependence of the binding arises from a more normal situation where imidazole and its anion can both bind competitively with solvent hydroxyls at the metal of the active site. This second model of binding is *formally* identical with that of Scheme I (with eq 2) in that it arises by the interchange of the labels E and L. The protonated form of the enzyme (EH) now is considered to bind to either the neutral (LH) or the anionic (L) forms of imidazole, whereas before the protonated (neutral) form of imidazole (LH) was considered to bind to either acidic (EH) or basic (E) forms of the active site. This model is shown in Scheme II and is designated BL [cf. case C of Bertini and Luchinat (1983)]. The linkage relation for this model is given by eq 4. Derivation of the expression for the apparent pK_i

$$pK_a^{EHL} - pK_a^L = pK_{LH}^{EH} - pK_L^{EH} \qquad (4)$$

for Scheme II leads to a formally identical expression to eq 3, except that the ionization of the complex is denoted by K_a^{EHL} to emphasize its attribution to a ligand ionization ("pyrrole" nitrogen deprotonation) within the enzyme–inhibitor complex. *The pH dependence of binding and spectral changes thus cannot differentiate between these models* that assume totally different physical bases for the pH dependence.

The spectral perturbations seen in the enzyme–inhibitor adducts can be interpreted within the BL model as being due to a change in coordination from complexation with neutral imidazole to complexation with the imidazole anion. The possibility of binding through the deprotonated "pyrrole" nitrogen has not received adequate attention in the earlier studies in view of the rather high (above 14) pK_a value for this ionization in imidazole (Sundberg & Martin, 1974), though not in the other analogues (Alberti et al., 1981). The unfavorably high pK_a in imidazole is compensated by a better coordination potential (greater ligand field strength), and the likelihood of coordination greatly improves if other stabilizing interactions such as chelation occur (Sundberg & Martin, 1974; Sigel & Martin, 1982). It is reasonable to speculate in the latter regard that an active site hydrogen bond donor, such as the NH group of Thr-199, interacts with a ring nitrogen of the imidazole anion, while the other nitrogen coordinates to the metal. Such an interaction cannot occur in neutral imidazole, suggesting a possible origin for the observed differences in symmetry and coordination number between the two types of imidazole–enzyme complexes. Analogues in which the other nitrogen is methylated are already known to show extremely weak affinity for CA (Khalifah, 1971; Alberti et al., 1981).

Since our pH data are formally compatible with either Scheme I or Scheme II, the distinction between them must await independent studies that can resolve this question. At present, it surprisingly appears that the BL model of Scheme II may provide a better physical interpretation of all the solution data, such as the absence of paramagnetic effects on solvent relaxation and the tetrahedral nature of the spectra of the imidazole and triazole complexes at high pH. It provides a structural basis for interpreting the visible spectral changes seen in complexes with the cobalt enzymes. It also brings the binding of these compounds in line with the binding of all other inhibitors, such as the anions and sulfonamides, that have been extensively investigated and have been found to compete with solvent hydroxyls for binding at the open coordination site of the metal. Independent verification that the "pyrrole" nitrogen is indeed deprotonated in the complexes at the predicted pH ranges would be extremely useful.

The only remaining evidence to support the WBA model of Scheme I is the crystallographic interpretations of the low-resolution electron density maps (Kannan et al., 1977; Kannan, 1980). Two considerations should be kept in mind in this regard. First, a crystallographic demonstration of the presence of a coordinated water molecule in the inhibitor complex cannot distinguish whether such a solvent ionizes in the complex. The simultaneous presence of a *nonionizing* water ligand is not excluded by the BL model at either low or high pH, although some of the spectra indicate that complexes are tetrahedral and are incompatible with pentacoordination. Second, the crystallographic studies on the imidazole complex were carried out at pH 8.7 in a high ammonium sulfate environment. This pH is sufficiently close to where the BL model predicts the coexistence of two different coordination modes for imidazole, a possibility not taken into account during the interpretation of the difference maps.

The considerations discussed above have important ramifications for the catalytic mechanism of this enzyme. The early stop-flow work on imidazole inhibition demonstrating competitiveness was done only at pH 7 (Khalifah, 1971). In view of the possible differences in the structure of the low- and high-pH complexes, a kinetic investigation of competitiveness at high pH and an extension of crystallographic studies of the

imidazole–CA I complex to pH 7 and pH 10 would both be clearly invaluable. *It may well be that only neutral imidazole is competitive with CO_2.* This view finds indirect support from the findings of Lindskog et al. (1984) that tetrazole and 1,2,4-triazole, both of which we predict (BL model) to bind in the anionic form at neutral pH and above, do not show competitiveness with CO_2. The realization that imidazole may indeed compete with hydroxyl for binding to the protonated form of the active site has implications to the proposed kinetic mechanisms of the enzyme in another way. It has been noted (Khalifah, 1980; Silverman & Vincent, 1983) that earlier studies assumed that CO_2 binding is pH independent by analogy with imidazole (Khalifah & Edsall, 1972; Khalifah, 1980), while recent work that recognizes exchange kinetics and solvent isotope effects has assumed that CO_2 binds only to the basic form of the active site [cf. Simonsson et al. (1979)]. Although the major basis for objections (Khalifah, 1980) to the latter assumptions is now obviated by our present results, independent support for a pH-dependent CO_2 binding is still lacking. *No inhibitor or substrate has ever been shown to bind to only the basic form of the enzyme.*[5] Until the actual mode of CO_2 binding has been determined, this question should be regarded as one of the major outstanding problems in the investigation of the catalytic mechanism of this highly efficient enzyme.

References

Alberti, G., Bertini, I., Luchinat, C., & Scozzafava, A. (1981) *Biochim. Biophys. Acta 668*, 16–26.

Bauer, R., Limkilde, P., & Johansen, J. (1977) *Carlsberg Res. Commun. 42*, 325–339.

Bertini, I., & Luchinat, C. (1983) *Acc. Chem. Res. 16*, 272–279.

Bertini, I., & Luchinat, C. (1984) *Adv. Inorg. Biochem. 6*, 71–111.

Bertini, I., Lanini, G., Luchinat, C., & Raciti, A. (1984) *Inorg. Chim. Acta 91*, 173–177.

Blackburn, G. M., Mann, B. E., Taylor, B. F., & Worrall, A. F. (1985) *Eur. J. Biochem. 153*, 553–558.

Campbell, I. D., Lindskog, S., & White, A. I. (1974) *J. Mol. Biol. 90*, 469–489.

Campbell, I. D., Lindskog, S., & White, A. I. (1975) *J. Mol. Biol. 98*, 597–614.

Hunt, J. B., Rhee, M., & Storm, C. B. (1977) *Anal. Biochem. 79*, 614–617.

Kanamori, K., & Roberts, J. D. (1983) *Biochemistry 22*, 2658–2664.

[5] Simonsson et al. (1982) have proposed that phenol, the CO_2 competitive inhibitor of CA II, binds only to the basic form of the enzyme on the basis that it is kinetically competitive with CO_2. As in the case of imidazole, the pH dependence of phenol binding cannot prove this, and our present work emphasizes the need to *independently* establish the protonation state of the bound phenol before conclusions can be firmly drawn.

Kannan, K. K. (1980) in *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) pp 184–205, Springer-Verlag, Berlin.

Kannan, K. K., Petef, M., Fridborg, H., Cid-Dresdner, H., & Lovgren, S. (1977) *FEBS Lett. 73*, 115–119.

Khalifah, R. G. (1971) *J. Biol. Chem. 246*, 2561–2573.

Khalifah, R. G. (1977) *Biochemistry 16*, 2236–2240.

Khalifah, R. G. (1980) in *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) pp 206–215, Springer-Verlag, Berlin.

Khalifah, R. G., & Edsall, J. T. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 172–176.

Khalifah, R. G., & Morley, P. J. (1984) *Arch Biochem. Biophys. 232*, 632–639.

Khalifah, R. G., Strader, D. J., Bryant, S. H., & Gibson, S. M. (1977) *Biochemistry 16*, 2241–2247.

Lindskog, S. (1982) *Adv. Inorg. Biochem. 4*, 115–170.

Lindskog, S. (1983) in *Zinc Enzymes, Metal Ions in Biology* (Spiro, T. G., Ed.) pp 78–121, Wiley-Interscience, New York.

Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O., & Strandberg, B. (1971) *Enzymes (3rd Ed) 5*, 587–665.

Lindskog, S., Engberg, P., Forsman, C., Ibrahim, S. A., Jonsson, B.-H., Simonsson, I., & Tibell, L. (1984) *Ann. N.Y. Acad. Sci. 429*, 61–75.

Maren, T. H. (1967) *Physiol. Rev. 47*, 595–781.

Maren, T. H. (1976) *Annu. Rev. Pharmacol. Toxicol. 16*, 309–327.

Riepe, M. E., & Wang, J. H. (1968) *J. Biol. Chem. 243*, 2779–2787.

Sigel, H., & Martin, R. B. (1982) *Chem. Rev. 82*, 385–426.

Silverman, D. N., & Vincent, S. H. (1983) *CRC Crit. Rev. Biochem. 14*, 207–255.

Simonsson, I., Jonsson, B.-H., & Lindskog, S. (1982) *Biochem. Biophys. Res. Commun. 108*, 1406–1412.

Stein, P. J., Merill, S. P., & Henkens, R. W. (1977) *J. Am. Chem. Soc. 99*, 3194–3196.

Sundberg, R. J., & Martin, R. B. (1974) *Chem. Rev. 74*, 471–517.

Taylor, P. W., King, R. W., & Burgen, A. S. V. (1970) *Biochemistry 9*, 3894–3902.

Tibell, L., Forsman, C., Simonsson, I., & Lindskog, S. (1984) *Biochim. Biophys. Acta 789*, 302–310.

Tibell, L., Forsman, C., Simonsson, I., & Lindskog, S. (1985) *Biochim. Biophys. Acta 829*, 202–208.

Valentine, J. S., & Pantoliano, M. W. (1983) in *Zinc Enzymes, Metal Ions in Biology* (Spiro, T. G., Ed.) pp 292–358, Wiley-Interscience, New York.

Whitney, P. L. (1970) *Eur. J. Biochem. 16*, 126–135.

Williams, T. J., & Henkens, R. W. (1985) *Biochemistry 24*, 2459–2462.

Wolpert, H. R., Strader, C. D., & Khalifah, R. G. (1977) *Biochemistry 16*, 5717–5721.

Wyman, J. (1964) *Adv. Protein Chem. 4*, 224–286.