Thermodynamics of Binding of the CO_2-Competitive Inhibitor Imidazole and Related Compounds to Human Carbonic Anhydrase I: An Isothermal Titration Calorimetry Approach to Studying Weak Binding by Displacement with Strong Inhibitors†

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Received July 9, 1992; Revised Manuscript Received December 14, 1992

ABSTRACT: The visible spectrum of Co(II)-substituted human carbonic anhydrase I (HCA I) complexed with the unique CO_2-competitive inhibitor imidazole undergoes a marked alkaline intensification, with a midpoint near pH 8 [Bauer, R., Limkilde, P., & Johansen, J. T. (1977) *Carlsberg Res. Commun. 42,* 325–339]. This change was first attributed to the ionization of a nondisplaced water ligand of the active-site metal in a five-coordinate complex. Later proposals favored assigning it to the deprotonation of the bound imidazole itself to give a tetrahedrally coordinated imidazolate anion at high pH. We have determined by isothermal titration calorimetry the pH dependence of the enthalpy of binding of imidazole and its analogues to HCA I and Co(II)HCA I. We devised an indirect strategy whereby the enthalpy of binding of the strong sulfonamide inhibitor methazolamide was determined in the absence and presence of a constant high concentration of the competing imidazole or its analogues. The standard enthalpy of binding of deprotonated methazolamide to the "acid" form of HCA I and Co(II)HCA I was found to be pH independent over the pH range of 6.5–9.5, as expected. It was also identical for both the zinc (−13.5 ± 1.1 kcal M^{-1}) and the cobalt (−13.7 ± 0.4 kcal M^{-1}) forms. The standard enthalpy of binding of neutral imidazole (average value −6.1 ± 0.8 kcal M^{-1}) surprisingly did not show any marked pH dependence, varying by about 1.1 and 2.6 kcal M^{-1} for the zinc and cobalt enzymes, respectively. The standard enthalpy of binding of the anionic forms of 1,2,3-triazole (−3.8 ± 0.2 kcal M^{-1}), 1,2,4-triazole (−6.5 ± 0.4 kcal M^{-1}), and tetrazole (−5.2 ± 0.5 kcal M^{-1}) was found to be pH independent, but in this case no pH-dependent spectral transition had been expected. The absence of appreciable pH changes in the standard enthalpy of binding of imidazole may be due either to the absence of enthalpically significant structural changes in the complex or to compensating heats of binding and ionization that nearly cancel out.

The mode of binding of the substrate CO_2 in the active site of carbonic anhydrase (EC 4.2.1.1) remains to be elucidated some 60 years after the discovery of this zinc metalloenzyme [for recent reviews see Eriksson and Liljas (1991) and Khalifah and Silverman (1991)]. No specific active-site side-chain or main-chain atom has been implicated in the binding of CO_2, and it is not known whether this substrate interacts with the zinc. It appears unlikely that crystallographic approaches will succeed in observing a CO_2–enzyme complex (Khalifah & Silverman, 1991; Khalifah et al., 1991), even though refined structures are now available for the major isozymes I, II, and III. Independent spectroscopic or other methods for determining substrate CO_2 binding have not been successful. Most recent speculation about the CO_2 binding site has come from molecular mechanics and molecular dynamics simulations (Liang & Lipscomb, 1990, 1991; Merz, 1990, 1991) that are at present not possible to verify by experiment. Site-specific mutagenesis experiments have been initiated that could shed significant light on substrate binding (Fierke et al., 1991; Nair et al., 1991; Alexander et al., 1991), but these are hampered by the inability to independently measure CO_2 binding affinity.

In view of the above, understanding the mode of binding of CO_2-competitive inhibitors of the enzyme becomes of considerable significance. Only two such inhibitors have been identified to date, these being imidazole for CA I (Khalifah, 1971) and phenol for CA II (Simonsson et al., 1982). Since the discovery of the competitiveness of imidazole, several studies have been carried out on its interaction with human CA I. These include a low-resolution crystallographic observation of the CA I–imidazole complex (Kannan et al., 1977), as well as several spectroscopic studies in which cobalt-substituted CA I was utilized to take advantage of the visible absorption spectrum of this catalytically active metalloenzyme (Bauer & Limkilde, 1977; Alberti et al., 1981; Khalifah et al., 1987). The visible spectrum of uninhibited Co(II)CA is strongly pH dependent, reflecting the ionization of the water–hydroxide ligand of the metal that is believed to be the nucleophile in the catalysis (Silverman & Lindskog, 1988):

$$\geq M{-}OH_2 \rightleftharpoons \geq M{-}OH^- + H^+$$

Most CA inhibitors are anions and bind by displacing this solvent ligand of the metal, leading to characteristic, pH-independent spectra that reflect the usually tetrahedral coordination environment of the cobalt metal (Bertini & Luchinat, 1983; Bertini et al., 1978):

$$\geq M{-}OH_2 + Inhib^- \rightleftharpoons \geq M{-}Inhib^- + H_2O$$

† Supported by the Medical Research Service of the Veterans Administration. A preliminary account of this work was presented at the joint meeting of the American Society of Biochemistry and Molecular Biology and the Biophysical Society in Houston, TX, February 9–13, 1992.

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0006-2960/93/0432-3058$04.00/0

Imidazole is nearly unique in that the visible spectrum of its inhibitor complex with Co(II)CA I is itself pH *dependent* (Bauer et al., 1977; Alberti et al., 1981). It undergoes a marked intensification at high pH with a midpoint for the change at about pH 8. Low-resolution crystallographic studies (Kannan et al., 1977) had suggested that imidazole binds within 3 Å of the zinc *without* displacing the water ligand of the metal ion,

$$\geq Zn{-}OH_2 \quad + \text{Imid} \rightleftharpoons \geq Zn\begin{matrix}H_2O\\ \diagdown\\ \text{Imid}\end{matrix}$$

and the affinity of imidazole to CA I was initially found to have no pH dependence (Wolpert et al., 1977). In view of this, it is not surprising that the pH-dependent spectral changes of the imidazole–Co(II)CA I complex were invariably interpreted as reflecting the ionization of an adjacent coordinated water ligand in a pentacoordinate complex:

$$\geq M\begin{matrix}H_2O\\ \diagdown\\ \text{Imid}\end{matrix} \rightleftharpoons \geq M\begin{matrix}OH^-\\ \diagdown\\ \text{Imid}\end{matrix} \quad + H^+$$

It was first pointed out by Bertini and Luchinat (1983) that the intense spectra of the imidazole complex with CA I at high pH are only consistent with a tetrahedral coordination, while at low pH the spectra reflect a five-coordinate or highly distorted four-coordinate complex. In other words, imidazole *must* be displacing the solvent ligand at high pH, so that the trigger for the pH-dependent spectral changes cannot be the ionization of an adjacent water ligand. Rather, the spectral changes may reflect the deprotonation of the imidazole (normal pK above 14) *within* the complex to yield a coordinated imidazolate anion (Bertini & Luchinat, 1983; Bertini et al., 1991; Khalifah et al., 1987, 1991):

$$\geq M\begin{matrix}H_2O\\ \diagdown\\ \text{Imid}\end{matrix} \rightleftharpoons \geq M{-}\text{Imid}^- + H_3O^+$$

An alternative possibility is to invoke an active-site group (other than the water ligand) as the ionizing group responsible for the spectral change, but this has not been seriously considered due to the paucity of candidate side chains with the requisite pK_a.

We have unambiguously shown by ^{15}N NMR and other properties (Rogers et al., 1987) that the metal ion of CA I is indeed capable of deprotonating and coordinating amide groups that have high pK_a's and metal affinity comparable to those of the imidazolate anion. Furthermore, we have thoroughly reinvestigated the binding of imidazole and its pH dependence and have computed the imidazole and imidazolate inhibition constants for the different binding models (Khalifah et al., 1987). Our analysis of the linkage between enzyme ionization and imidazole binding revealed that the pK for the spectral changes can be independently predicted from the pH dependence of the inhibition (Khalifah et al., 1987). Unfortunately, we found that *the binding data cannot be used to distinguish whether the ionization in the complex is due to an imidazole–imidazolate or a water–hydroxide ionization, since the proton balance is the same.* Similarly, we cannot exclude the involvement of an ionizing active-site side chain, although it appears quite unlikely that His-200 is involved.

In the continued absence of high-resolution crystallographic studies of the imidazole complex at low and high pH, it is apparent that *independent* approaches are needed to elucidate the binding modes of this important and unique competitive inhibitor (Khalifah, 1991). We now report microcalorimetric titration studies on the binding of imidazole and related inhibitors to HCA I. [An outstanding review of calorimetry and its applications in similar biochemical studies can be found in Eftink and Biltonen (1980) and references therein.] In particular, we have sought to detect possible pH-dependent transitions in the imidazole–CA complex that may be reflected in the enthalpy of binding. It should be noted that the microcalorimetric technique is equally applicable to the zinc and cobalt forms of the enzyme and is thus capable, in principle, of detecting whether this metal replacement in the active site influences the binding of imidazole. An important part of our study has been the determination of the enthalpy of binding of methazolamide, a pharmacologically important sulfonamide inhibitor of CA. We are aware of only one previous calorimetric investigation of inhibitor binding to CA that focused on isozyme II inhibition by sulfonamides and cyanate (Binford et al., 1974). Our present work has general relevance to the problem of the measurement of the enthalpy of binding of relatively weak protein ligands. Such inhibitors must be added from a very concentrated solution in order to achieve saturation, and the heats of dilution can, as in the case of imidazole, dominate over the intrinsic binding heat change that is being sought. To overcome this, we utilized a *differential* or *competitive* approach in which the enthalpy of binding of a strong sulfonamide inhibitor (methazolamide) was measured in the absence and presence of weaker competing inhibitors.

MATERIALS AND METHODS

Chemicals. Imidazole (Grade III, 99+%), Bis-tris propane and methazolamide were purchased from Sigma Chemical Co. 1,2,4-Triazole and tetrazole (sublimed, 99+%) were obtained from Aldrich. 1,2,3-Triazole was obtained from Alfa Products. Ultrapure cobalt sulfate was a product of Johnson-Matthey.

Enzymes. Human carbonic anhydrase I (formerly isozyme B) was prepared from freshly outdated erythrocytes using affinity chromatography on CM-Bio-Gel A to which *p*-(aminomethyl)benzenesulfonamide had been attached (Khalifah et al., 1977). ApoHCA I was prepared by removal of the zinc using the pyridine-2,6-dicarboxylate method (Hunt et al., 1975). Co(II)CA I was prepared either by dialyzing apoCA I against an excess of $CoSO_4$ or by direct addition of $CoSO_4$. The degree of metal incorporation was monitored spectrally by taking aliquots into excess acetazolamide, since this inhibitor complex has a high visible extinction coefficient.

Spectrophotometry. UV–Visible spectra were taken on a Hewlett-Packard HP8452A photodiode array spectrophotometer controlled by a Dell 310 computer. Temperature control was maintained by an external circulating water bath or by a thermoelectric accessory. Spectrophotometric pH titrations studies were carried out at different temperatures in our work on Co(II)HCA I and methazolamide. In such experiments, Tris buffers were used and the pH was computed at different temperatures by using the accurately known value of the enthalpy of ionization of Tris.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) (Sturtevant, 1972; Biltonen & Langerman, 1979) was carried out using the Omega instrument and ORIGIN software from Microcal, Inc. (Northampton, MA). The calorimeter was controlled by an IBM PS/2 Model 30-286 computer. A detailed description of this differential microcalorimeter has been published (Wiseman et al., 1989). The sample cell has a volume of 1.42 mL. The solution to be added is placed in a 250-μL injection syringe whose needle is paddle-shaped and rotated at 400 rpm. The syringe plunger

is mechanically coupled to a digital stepping motor that is computer-controlled for precise injections. The cell containing enzyme was equilibrated at 24.6 ± 0.6 °C prior to starting the injections of microliter volumes of ligand solution. Each injection caused heat to be evolved or absorbed that was measured by integration of the pulse. Typical titrations utilized 20–50 additions (pulses). The integrated heats produced by such pulses were then fitted by the ORIGIN software to a 1:1 ligand binding model to obtain the total heat evolved or absorbed upon complex formation. The software also provides estimates of the binding affinity and the ligand-to-protein stoichiometry at full binding, which can serve as an internal check of accuracy. Molar reaction enthalpies reported in this study were computed by normalizing on the known protein concentration in the cell rather than on the added ligand concentration. All experiments were carried out in triplicate. The instrument used internal electric calibration of the heat generated, and this could be monitored at various stages by computing the known heats of protonation of various ionizable buffer and ligand species that were titrated. All enthalpy data are reported in units of kilocalories per (moles per liter) and can be converted to kilojoules per (moles per liter) by multiplication by the factor 4.184.

Preparation of Samples for Calorimetry. HCA I enzyme samples (ca. 0.15–0.20 mM) were dialyzed first against the appropriate buffers in the cold. The samples were then dialyzed against buffer solutions (15 mM Bis-tris propane and 0.067 M K_2SO_4) containing the required concentration of imidazole (usually 0.25 M) or other inhibitor at room temperature. After equilibration, the inhibitor/buffer dialysate solution was used to dissolve the extremely strong inhibitor methazolamide at a typical concentration of 4 mM. In the case of Co(II)HCA I, dialysis against imidazole, a good metal ligand, was avoided, so that solutions were made by direct addition of imidazole from a concentrated solution. Due to a limited amount of 1,2,3-triazole, this inhibitor was directly added into the appropriate enzyme solutions. The pH was monitored at all stages and adjusted if necessary. The calorimetric experiment was then carried out by placing the imidazole-inhibited solution in the 1.42-mL sample cell and then titrating it by injecting the methazolamide/imidazole solution in a sequence of 6-μL pulses spaced 3 min apart to ensure thermal reequilibration. Control and reference experiments were carried out on the enthalpy of binding of methazolamide as described above, except that imidazole or other inhibitors were omitted. The heats of ionization of all buffer and inhibitor/buffer solutions were also routinely measured by titrating them with 40 mM NaOH at an ionic strength of 0.2 M made with K_2SO_4.

Molecular Graphics. Examination of portions of the active site of CA and simple manipulations to visualize bound inhibitors were carried out using the Alchemy III software (Tripos Assoc., St. Louis, MO). Crystallographic coordinates for human CA I and II were obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977).

RESULTS

van't Hoff Enthalpy of Co(II)HCA I Ionization. The ionization enthalpy, $\Delta H'_e$, of the active-site group that controls inhibitor binding, presumably the water ligand of the metal, is needed in the analysis of the enthalpy of binding of the inhibitors that we studied (vide infra). To calculate this value, we determined the temperature dependence of the pK_a of this ionization from the well-known visible spectral changes that accompany the titration of Co(II)HCA I [cf. Bertini and Luchinat (1983)]. We found that $\Delta H'_e$ is 8.9 ± 0.6 kcal M^{-1} and the pK_e is 7.41 ± 0.04 at 25 °C (Table I). A very rough estimate of 6.9 kcal M^{-1} ($\Delta pK/\Delta T \approx -0.02$) for the corresponding ionization in bovine CA isozyme II has been given by Pocker and Stone (1968), based on the temperature dependence of esterase activity pH profiles.

Table I: Ionization Enthalpies and pK_a Values of Inhibitors and Enzymes at 298 K Utilized in Calculations[a]

compound	ionization	pK'_a	$\Delta H'_{ion}$ (kcal M^{-1})
imidazole	$pK_{imid,1}$ ("pyridine" N)	7.06[b]	9.03[b]
imidazole	$pK_{imid,2}$ ("pyrrole" N)	14.44[b]	17.6[b]
1,2,3-triazole	$pK_{123T,2}$ ("pyrrole" N)[c]	9.42[d]	7.9 ± 0.1
1,2,4-triazole	$pK_{124T,2}$ ("pyrrole" N)[c]	10.26[d]	9.2 ± 0.1
tetrazole	$pK_{Tet,2}$ ("pyrrole" N)	4.89[d]	2.5 ± 0.1
methazolamide	Sulfonamido N (pK_s)	7.26 ± 0.03	7.0 ± 0.1
Co(II)HCA I	water ligand (pK_e)	7.41 ± 0.04	8.9 ± 0.6
Zn(II)HCA I	water ligand (pK_e)	(7.41)[e]	(8.9)[e]

[a] All calorimetric measurements were carried out at 24.6 ± 0.6 °C in solutions that were of 0.2 M ionic strength made up with K_2SO_4. pK_a values refer to 25 °C and an ionic strength of 0.2 M whenever available. [b] Izzat and Christensen (1976). [c] Protonation of neutral triazoles and tetrazole (pK_1 of "pyridine" N) is not significant except below about pH 3. [d] Alberti et al. (1981). [e] Assumed to be the same as Co(II)HCA I.

Ionization Enthalpies of Inhibitors and Buffers. The majority of the inhibitors studied had an ionization whose $\Delta H'_{ion}$ had to be taken into account in our calculations. For those compounds for which we could not readily find a literature value for $\Delta H'_{ion}$, we determined approximate values by calorimetry. The data obtained in this study or utilized from the literature are listed in Table I. A spectrophotometric titration was also carried out on the sulfonamide group ionization of the inhibitor methazolamide under our experimental conditions, using the pH dependence of the absorbance difference between 280 and 350 nm (Table I). Note that the ionization listed for tetrazole and the triazoles is deprotonation to give the corresponding anions ("pyrrole nitrogen" ionization). Two ionizations are listed for imidazole, the deprotonation of the imidazolium cation to give the neutral imidazole (at the "pyridine" nitrogen) and deprotonation of neutral imidazole to give the imidazolate anion (at the "pyrrole" nitrogen):

Similarly, determinations were necessary for the heats of ionization of the buffers used. In experiments with inhibitors, the buffering capacity was altered by the presence of ionizable inhibitors at the concentrations and pH values used. Consequently, we determined by calorimetry the experimental or "apparent" heat evolved, for each buffer and buffer/inhibitor solution used, by titration with several small aliquots of NaOH. The computed "apparent" enthalpies of ionization are provided as supplementary material. This empirical procedure was especially convenient for Bis-tris propane due to the complex dibasic nature of this buffer. Note that the enthalpy of proton dissociation or ionization ($\Delta H'_{buf}$) and the enthalpy of reaction with OH^- ($\Delta H'_{OH}$) are related by the enthalpy of water ionization $\Delta H'_{wat}$, taken as 13.337 kcal M^{-1} (Izzat & Christensen, 1976), since $\Delta H'_{buf} = \Delta H'_{OH} + \Delta H'_{wat}$.

Apparent Enthalpy of Binding of Methazolamide to HCA I and Co(II)HCA I. The titration of HCA I and Co(II)HCA I with the small aliquots of the sulfonamide inhibitor methazolamide produces readily measurable heat changes (Figure 1A,B). As noted above, the analysis with the ORIGIN

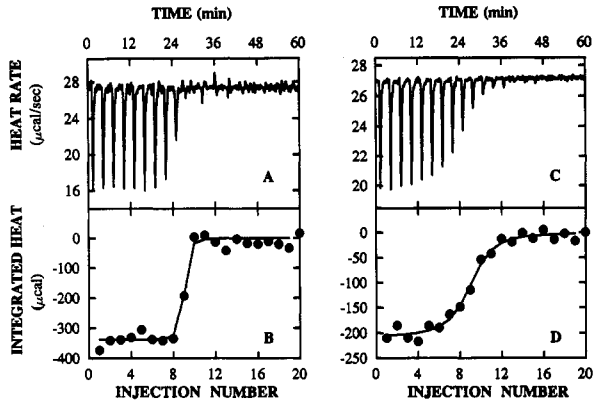

FIGURE 1: Typical isothermal titration calorimetry traces (A and C) and the analysis (B and D) of the integrals of the pulses produced by adding small increments of methazolamide to human carbonic anhydrase I (see text). Figures compare the results in the absence (A and B) and presence (C and D) of 0.25 M imidazole. The markedly sharper changes seen in panels A and B compared to panels C and D reflect the weakened affinity in the latter for methazolamide due to the competing imidazole.

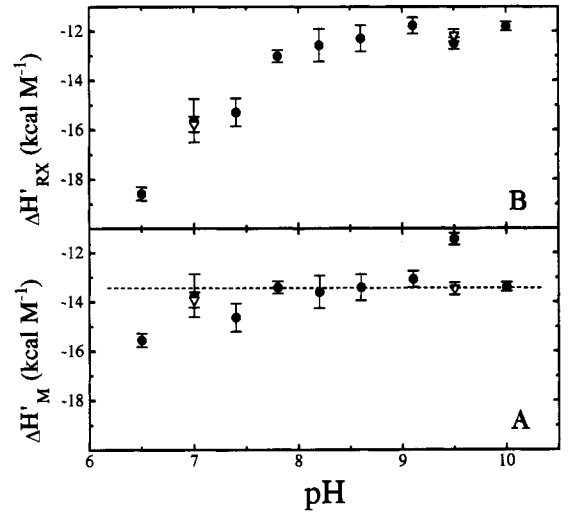

FIGURE 2: (A) Standard enthalpy for intrinsic binding of methazolamide to Zn(II) (filled circles) and Co(II) (open triangles) human carbonic anhydrase I as a function of pH. The data refer to the binding of the deprotonated methazolamide to the acid (zinc–water) form of the enzyme. (B) Actual observed reaction enthalpies for binding of methazolamide to HCA I. At each pH these observed reaction enthalpies were corrected for the heats of ionization of methazolamide, enzyme, and buffer to yield the results in panel A (see text). The dashed line is at the average value (−13.5 ± 1.1 kcal M^{-1}) of the data for HCA I in panel A. All results were obtained at 24.6 °C in Bis-tris propane sulfate buffers of ionic strength 0.2 M.

software also computes the apparent equilibrium constant for binding and the stoichiometry of binding (ratio of ligand to enzyme in complex). The latter was a useful internal check on sample integrity and methodology and was typically 1.0 ± 0.1. In view of the extremely high affinity of methazolamide for CA I (Maren & Sanyal, 1983), particularly in the absence of competing inhibitors, the uncertainty in the computed equilibrium constant was much too large for the K_i values to be quantitatively useful. The apparent reaction enthalpies for methazolamide binding, $\Delta H'_{RX}$, are shown in Figure 2B. A pH range from 6.5 to 10.0 was investigated for HCA I, and Co(II)HCA I was studied at pH 7.0 and 9.5. These experimental $\Delta H'_{RX}$ values are seen to be more negative at lower pH values. However, as discussed below, their interpretation in terms of *intrinsic* binding enthalpies (Figure 2A) requires definition of a reference binding reaction and

Table II: Apparent (Reaction) Enthalpy of Binding of Methazolamide to HCA I and Co(II)HCA I in the Presence of Competing Inhibitors[a]

			ΔH_{RXI} (kcal M^{-1})	
inhibitor	concn (M)	pH	HCA I	Co(II)HCA I
imidazole	0.25	9.50	−8.8 ± 0.3[b]	−7.8 ± 0.3
imidazole	0.25	9.25	−9.6 ± 0.1[c]	
imidazole	0.25	9.10	−7.4 ± 0.1	
imidazole	0.25	8.60	−8.8 ± 0.3	−8.6 ± 0.3
imidazole	0.25	8.20	−8.2 ± 0.6	
imidzole	0.25	7.80	−9.2 ± 0.2	−8.2 ± 0.3
imidazole	0.25	7.40	−10.1 ± 0.3	
imidazole	0.25	7.00	−10.7 ± 0.4	−11.0 ± 0.2
imidazole	0.25	6.50	−11.9 ± 0.3	−12.7 ± 0.1
1,2,3-triazole	0.10	8.31	−9.0 ± 0.1	
1,2,3-triazole	0.10	7.27	−9.2 ± 0.1	
1,2,4-triazole	0.25	9.10	−7.0 ± 0.1	
1,2,4-triazole	0.25	7.40	−6.8 ± 0.5	
tetrazole	0.050	8.00	−9.4 ± 0.4	
tetrazole	0.050	6.50	−9.9 ± 0.5	

[a] Unless noted otherwise, data are for 24.6 ± 0.6 °C and Bis-tris propane buffers of 0.2 M ionic strength made up with K_2SO_4. Experimental molar enthalpies are not corrected for any ionization reactions. [b] Dimethylglycine hydrochloride buffer. [c] Sodium barbital buffer.

subsequent calculation of contributions arising from ionizations due to enzyme, methazolamide, and buffer species. A thorough discussion of the thermodynamics of such coupled reactions and ionizations can be found in Eftink and Biltonen (1980).

Apparent Enthalpy of Binding of Methazolamide in the Presence of Competing Inhibitors. The ITC measurements of the apparent enthalpies of binding of methazolamide to HCA I and Co(II)HCA I were also carried out in the presence of the inhibitor imidazole or some of its analogues. These inhibitors compete with methazolamide for binding to enzyme. The methazolamide apparent reaction enthalpies obtained in this way are designated as $\Delta H'_{RXI}$ and are summarized in Table II. The imidazole analogues studied were 1,2,3- and 1,2,4-triazole and tetrazole. Comparison of the less-steep profiles in Figure 1C,D with those of Figure 1A,B clearly shows that the methazolamide binding has been weakened in presence of the imidazole, i.e., that imidazole and the other inhibitors compete with methazolamide as expected. A direct verification of this was obtained by examining the visible spectrum of Co(II)HCA I in the presence of imidazole, methazolamide, and a mixture of the two (supplementary material). The difference between the $\Delta H'_{RXI}$ values and the $\Delta H'_{RX}$ values contains information on the *intrinsic* enthalpies of binding of the weaker inhibitors. This can only be obtained after taking into account contributions from the enthalpies of ionization of enzyme, methazolamide, buffer, and the inhibitors, as well as corrections for the incomplete saturation of the enzyme by the weaker inhibitors prior to addition of the methazolamide. A sequence of reactions then must be explicitly assumed to occur in order to extract intrinsic enthalpies that refer to binding of thermodynamic states of ligand and enzyme in defined protonation states [cf. Biltonen and Langerman (1979) and Eftink and Biltonen (1980)]. Such an analysis is discussed below for each type of experiment carried out.

THERMODYNAMIC ANALYSES

Enthalpy of Binding of Methazolamide to HCA I and Co(II)HCA I. Sulfonamides are believed to bind at equilibrium as the deprotonated anions to the "acid" (formally zinc–water) form of carbonic anhydrase by displacing the

Scheme I: Methazolamide Binding to HCA-I

(1) $EOH + H^+ \xrightarrow{-\Delta H'_e f_e} EOH_2$

(2) $RSO_2NH_2 \xrightarrow{\Delta H'_s(1-f_s)} RSO_2NH^- + H^+$

(3) $EOH_2 + RSO_2NH^- \xrightarrow{\Delta H'_M} E\text{-}RSO_2NH^- + H_2O$

(4) $BH^+ \xrightarrow{\Delta H'_{buf}(f_e+f_s-1)} B + H^+$

water–hydroxide solvent ligand of the metal ion [cf. Rogers et al. (1987) and references therein]. We have consequently computed the intrinsic enthalpy ($\Delta H'_M$) for this binding reaction using the sequence of reactions that are detailed in Scheme I. These reactions specify that the fraction of enzyme in the basic form, f_e, must be protonated (line 1 of Scheme I) and the fraction of sulfonamide in its acid form, $1-f_s$, must be deprotonated (line 2) for complete binding to occur (line 3), with buffer donating or receiving protons as needed in each reaction (line 4). We note that Scheme I involves standard enthalpy changes from three ionizations belonging to enzyme (pK_e), methazolamide (pK_s), and buffer, as well as the standard enthalpy from one binding reaction that goes to completion. We adopt the convention of referring to the enthalpies of proton *dissociations* (ionizations) by lowercase subscripts and the enthalpies of inhibitor binding (*association* steps) by uppercase subscripts. The deprotonation enthalpies of free enzyme and free methazolamide are $\Delta H'_e$ and $\Delta H'_s$. The concentration of buffer is such that it will accept or donate all protons that are given off or taken up. The observed reaction enthalpy $\Delta H'_{RX}$ must then be the sum of the molar enthalpies of each step in Scheme I after weighting by the appropriate fractions of ionized species taking part in that step:

$$\Delta H'_{RX} = -\Delta H'_e f_e + \Delta H'_s(1-f_s) + \Delta H'_M + \Delta H'_{buf}(f_e + f_s - 1) \quad (1)$$

We can then solve for $\Delta H'_M$, the *pH-independent* intrinsic enthalpy of binding of deprotonated methazolamide to the "acid" (EOH_2) form of CA I:

$$\Delta H'_M = \Delta H'_{RX} + f_e(\Delta H'_e - \Delta H'_{buf}) + (1-f_s)(\Delta H'_{buf} - \Delta H'_s) \quad (2)$$

By using eqs 1 and 2 we have computed $\Delta H'_M$ for both HCA I and Co(II)HCA I over the pH range of 6.5–10. The results are plotted in Figure 2A, and the magnitude of the corrections can be appreciated by comparison to the $\Delta H'_{RX}$ plotted in Figure 2B. It can be seen that there is no significant pH dependence of $\Delta H'_M$ for either CA I (filled circles) or Co(II)CA I (open triangles). Furthermore, the zinc and cobalt forms show no significant difference, with the average $\Delta H'_M$ being -13.5 ± 1.1 kcal M^{-1} for HCA I and -13.7 ± 0.4 kcal M^{-1} for Co(II)HCA I over the pH ranges studied.

Enthalpy of Binding of Imidazole to HCA I and Co(II)-HCA I. The availability of an accurate and pH-independent value for the intrinsic enthalpy of binding of methazolamide ($\Delta H'_M$) provides a means of interpreting the experimental heats of reaction ($\Delta H'_{RXI}$) obtained in the presence of competing imidazole and given in Table II. Scheme II describes the sequence of reactions for computing the enthalpy of binding of neutral imidazole to the "acid" form (EOH_2) of CA I in the *limiting case* (modified below) where methazolamide is used to displace imidazole that is assumed to be

Scheme II: Methazolamide Binding to HCA-I in the Presence of Saturating, Competing Imidazole

(1) $E\text{-}H_2O\text{-}Imid \xrightarrow{-\Delta H'_{IMID}} EOH_2 + Imid$

(2) $RSO_2NH_2 \xrightarrow{\Delta H'_s(1-f_s)} RSO_2NH^- + H^+$

(3) $EOH_2 + RSO_2NH^- \xrightarrow{\Delta H'_M} E\text{-}RSO_2NH^- + H_2O$

(4) $Imid + H^+ \xrightarrow{-\Delta H'_{imid,1}(1-f_{imid,1})} ImidH^+$

(5) $BH^+ \xrightarrow{\Delta H'_{buf,i}[(1-f_{imid,1})-(1-f_s)]} B + H^+$

at high enough concentration to completely saturate the enzyme. The experimental $\Delta H'_{RXI}$ is the sum of the contributions of the five steps shown in Scheme II, as given by eq 3. Here $f_{imid,1}$ is the fraction of neutral (nonprotonated) imidazole and the enthalpy of deprotonation of the imidazolium cation is $\Delta H'_{imid,1}$:

$$\Delta H'_{RXI} = -\Delta H'_{IMID} + \Delta H'_s(1-f_s) + \Delta H'_M - \Delta H'_{imid,1}(1-f_{imid,1}) + [(1-f_{imid,1}) - (1-f_s)]\Delta H'_{buf,i} \quad (3)$$

We can solve for $\Delta H'_{IMID}$, the intrinsic enthalpy of binding of neutral imidazole to the "acid" form of CA I, as shown:

$$\Delta H'_{IMID} = -\Delta H'_{RXI} + \Delta H'_M + (1-f_s)(\Delta H'_s - \Delta H'_{buf,i}) + (1-f_{imid,1})(\Delta H'_{buf,i} - \Delta H'_{imid,1}) \quad (4)$$

However, the 0.25 M imidazole concentrations used in our experiments of Table II were sufficient to inhibit CA I to only 67–85%, based on the known imidazole inhibition constant and its small pH dependence (Khalifah et al., 1987). Higher concentrations were not used in order to avoid possible denaturation of the enzyme at lower pH (Wolpert et al., 1977). If f_i is the fractional inhibition by imidazole at any given pH prior to methazolamide addition, then methazolamide will react with the enzyme fraction that is inhibited (f_i) according to Scheme II and with the fraction of enzyme that is free ($1-f_i$) according to Scheme I. The observed reaction heat will thus be given by

$$\Delta H'_{RX,obs} = f_i\Delta H'_{RXI} + (1-f_i)\Delta H'_{RX} \quad (5)$$

The fractional inhibition can be calculated from the K_i values of Khalifah et al. (1987) by using the relation $f_i = ([Imid]/K_i)/(1 + [Imid]/K_i)$, where $I_{total} = [Imid] + [ImidH^+] = [Imid]/(1 + [H^+]/K_i)$. We note that K_i, the inhibition dissociation constant for imidazole, was previously defined in terms of inhibition by the *neutral* form of imidazole, since protonated imidazole does not bind to CA I (Wolpert et al., 1979). By combining eqs 1–5 and the above relations, we can solve for the intrinsic enthalpy of binding of neutral imidazole to the "acid" form of CA I:

$$\Delta H'_{IMID} = (1-f_{imid})(\Delta H'_{buf,i} - \Delta H'_{imid,1}) + (1/f_i)[\Delta H'_M - \Delta H'_{RX,obs} + f_e(1-f_i)(\Delta H'_{buf,i} - \Delta H'_e) + (1-f_s)(\Delta H'_s - \Delta H'_{buf,i})] \quad (6)$$

The values of $\Delta H'_{IMID}$ obtained by this analysis are plotted in Figure 3A as a function of pH. It is immediately apparent that, within the accuracy of the data, the enthalpy of binding of imidazole to HCA I shows little (of the order of 1 kcal M^{-1}), if any, significant pH dependence. A somewhat larger (2.5 kcal M^{-1}) and possibly more significant variation is seen for Co(II)HCA I (Figure 3B). To illustrate these small changes and to obtain high- and low-pH limits (discussed

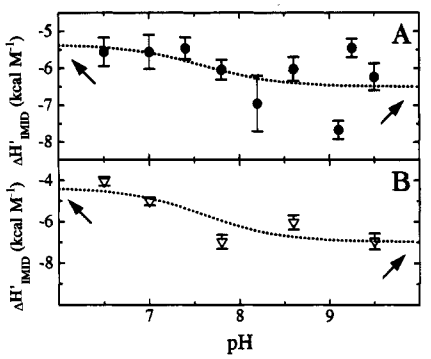

FIGURE 3: Standard enthalpies of binding of imidazole to Zn(II) (A) and Co(II) (B) human carbonic anhydrase I at 24.6 °C in Bis-tris propane buffers of 0.2 M ionic strength. The data are computed for the standard reaction of neutral imidazole with the acid (zinc–water) form of the enzyme after taking into account the heats of ionization of enzyme, imidazole, and imidazole-containing buffers (see text). The dotted line in panel A represents a nonlinear fit of the data to a simple titration function to illustrate the possible pH dependence. The pK_a of the fit was 7.6 ± 0.7 and the limits (arrows) were –5.4 ± 0.5 and –6.5 ● 0.2 kcal M^{-1} at low and high pH, respectively. A similar illustrative fit was done in panel B except that the pK_a was constrained to 7.6 for comparison with panel A. The limits were –4.4 ± 0.6 and –7.0 ± 0.5 kcal M^{-1} at low and high pH, respectively. Note that the data are derived from experiments where enzyme was titrated with methazolamide in the absence and presence of constant concentrations of imidazole.

Scheme III: Methazolamide Binding to HCA-I in the Presence of Saturating Inhibitors That Bind as Anions

(1) $E\text{-}I^- + H_2O \xrightarrow{-\Delta H'_{ANION}} EOH_2 + I^-$

(2) $RSO_2NH_2 \xrightarrow{\Delta H'_s(1-f_s)} RSO_2NH^- + H^+$

(3) $EOH_2 + RSO_2NH^- \xrightarrow{\Delta H'_M} E\text{-}RSO_2NH^- + H_2O$

(4) $H\text{-}I \xrightarrow{-\Delta H'_{ion}(1-f_{anion})} H^+ + I^-$

(5) $BH^+ \xrightarrow{\Delta H'_{buf,i}(f_s-1)} B + H^+$

further below), we have nevertheless fit the data of Figure 3A to a titration curve using nonlinear least-squares. A constrained fit was also carried out on the data in Figure 3B, with the parameters shown in the figure legends. The low-pH limiting values are taken to represent the binding of neutral imidazole to the acid form of CA I (Khalifah et al., 1987), yielding virtually identical $\Delta H'_{IMID}$ values of –5.4 ± 0.5 and –4.4 ± 0.6 kcal M^{-1} for the zinc and cobalt forms of HCA I, respectively.

Enthalpy of Binding of Imidazole Analogues to HCA I. The analogues 1,2,3- and 1,2,4-triazole and tetrazole are considerably more acidic than imidazole (Table I). They are believed to bind to the enzyme only in their anionic deprotonated form in the pH range of the present study (Khalifah et al., 1987; Tibell et al., 1985). Scheme III provides the standard model and the steps that are assumed to occur when methazolamide is added to enzyme fully saturated by the anionic forms of such competing inhibitors. The observed enthalpy of reaction in the presence of the anions, $\Delta H'_{RXA}$, will be equal to the sum of steps 1–5 of Scheme III after weighting by the appropriate fractions of deprotonated (anionic) inhibitors (f_{anion}, with an enthalpy of deprotonation of $\Delta H'_{ion}$) and methazolamide (f_s):

$$\Delta H'_{RXA} = \Delta H'_M - \Delta H'_{ANION} + (1-f_s)(\Delta H'_s - \Delta H'_{buf,i}) + (1-f_{anion})(\Delta H'_{buf,i} - \Delta H'_{ion}) \quad (7)$$

The intrinsic enthalpy of binding of the anionic forms of the inhibitors to the "acid" form of the enzyme will then be

$$\Delta H'_{ANION} = \Delta H'_M - \Delta H'_{RXA} + (1-f_s)(\Delta H'_s - \Delta H'_{buf,i}) + (1-f_{anion})(\Delta H'_{buf,i} - \Delta H'_{ion}) \quad (8)$$

As in the case of imidazole, if the inhibitors were initially present at a concentration that only inhibits the enzyme by a fraction f_i prior to titration with methazolamide, then the observed reaction enthalpy will be a weighted sum of the contributions from Schemes I and III:

$$\Delta H'_{RXA,obs} = f_i \Delta H'_{RXA} + (1-f_i)\Delta H'_{RX} \quad (9)$$

The corrected $\Delta H'_{ANION}$ can be solved for (eq 10) with a result analogous to that of eq 6:

$$\Delta H'_{ANION} = (1-f_{anion})(\Delta H'_{buf,i} - \Delta H'_{ion}) + (1/f_i)[\Delta H'_M - \Delta H'_{RXA,obs} + f_e(1-f_i)(\Delta H'_{buf,i} - \Delta H'_e) + (1-f_s)(\Delta H'_s - \Delta H'_{buf,i})] \quad (10)$$

The concentrations of tetrazole and triazoles used in our experiments (cf. Table II) were such that the corrections were quite small, since f_i values were greater than 0.93 at the worst. The corrected standard enthalpies of binding are given in Table III and are seen to be in the range of –4 to –7 kcal M^{-1}. The inhibitors were studied at two pH values each, and it can also be seen that there is no apparent pH dependence in the enthalpy of binding within the experimental errors.

Table III: Standard Enthalpy of Binding of Anionic Forms of Inhibitors to the Acid Form of HCA I[a]

inhibitor	pH	$\Delta H'_{ANION}$
tetrazole	8.00	–4.9 ± 0.5
tetrazole	6.50	–5.5 ● 0.5
1,2,4-triazole	9.10	–6.2 ± 0.1
1,2,4-triazole	7.40	–6.7 ± 0.5
1,2,3-triazole	8.31	–4.1 ● 0.1
1,2,3-triazole	7.27	–3.8 ± 0.1
imidazolate	6.5–10	~–15 ± 1[b]

[a] Data obtained at 24–25 °C in Bis-tris propane buffers of ionic strength 0.2 adjusted with K_2SO_4. [b] Computed using eq 11. See text for discussion.

Scheme IV: Binding of Methazolamide to HCA-I in the Presence of Saturating Imidazolate Anion

(1) $E\text{-}H_2O\text{-}Imid^- \xrightarrow{-\Delta H'_{IMID,A}} EOH_2 + Imid^-$

(2) $RSO_2NH_2 \xrightarrow{\Delta H'_s(1-f_s)} RSO_2NH^- + H^+$

(3) $EOH_2 + RSO_2NH^- \xrightarrow{\Delta H'_M} E\text{-}RSO_2NH^- + H_2O$

(4) $Imid^- + H^+ \xrightarrow{-\Delta H'_{Imid,2}} Imid$

(5) $Imid + H^+ \xrightarrow{-\Delta H'_{Imid,1}(1-f_{Imid,1})} ImidH^+$

(6) $BH^+ \xrightarrow{\Delta H'_{buf,i}[(1-f_{Imid,1})-(1-f_s)+1]} B + H^+$

Enthalpy of Binding of Imidazole as the Imidazolate Anion. Since there is spectroscopic evidence that the binding at high pH may be predominantly by the imidazolate anion, we also analyzed the previous imidazole data according to Scheme IV in which only the imidazolate anion is assumed to bind for simplicity. This scheme is strictly analogous to Scheme III for the analogues with the further inclusion of the heat of the first ionization of imidazole (pK_a 7) that occurs in the experimental pH range. Assuming a saturating concentration, the intrinsic enthalpy of binding of the imidazolate anion,

Table IV: Thermodynamics of Binding of Inhibitors to HCA I and Co(II)HCA I at 298 K[a]

inhibitor (assumed binding form)	pK'_a	$\Delta G'_{BIND}$ (kcal M^{-1})	$\Delta H'_{BIND}$ (kcal M^{-1})	$\Delta S'_{BIND}$ (cal M^{-1} K^{-1})
imidazolate (–)	14.44	–11.2	~–15.1 (–14.6)[b]	~–13 (~–11)[b]
1,2,4-triazole (–)	10.26	–8.3	–6.5	+6
1,2,3-triazole (–)	9.42	–7.1	–3.8	+11
imidazole (0)	7.06	–2.2	–5.4 (–4.4)[b]	–11 (–7)[b]
tetrazole (–)	4.89	–5.6	–5.2	+1
methazolamide (–)	7.26	–11.12[c]	–13.5 (–13.7)[b]	–8 (–9)[b]

[a] Data are average values over the pH ranges studied, except for imidazole, where the enthalpies are limits of fits to a titration function (see Discussion). All data were obtained at 24.6 ± 0.6 °C in Bis-tris propane buffers of 0.2 M ionic strength made up with K_2SO_4. Values are computed for the equilibrium where the acid (zinc–water) form of enzyme combines with the form of the ligand (anion –, neutral 0) indicated in column 1. [b] These values are for Co(II)HCA I. [c] Data of King and Maren (1974).

$\Delta H'_{IMID,A}$, would then be

$$\Delta H'_{IMID,A} = -\Delta H'_{RXI} + \Delta H'_M + (1 - f_s)(\Delta H'_s - \Delta H'_{buf,i}) + (1 - f_{imid,1})(\Delta H'_{buf,i} - \Delta H'_{imid,1}) + (\Delta H'_{buf,i} - \Delta H'_{imid,2}) \quad (11)$$

It will be recognized that, except for the last term, the right-hand side of eq 11 is identical to that of eq 4. Since we are only interested in the high-pH limiting values of the fits in Figure 3, we can use eqs 11 and 4 to express $\Delta H'_{IMID,A}$:

$$\Delta H'_{IMID,A} = (\Delta H'_{IMID})_{highpH} + (\Delta H_{buf,i} - \Delta H_{imid,2}) \quad (12)$$

The last term on the right-hand side is the difference between the heat of dissociation of the "pyrrole" proton of imidazole (about 17.6 kcal M^{-1}) and its donation to the buffer (about 9 kcal M^{-1}). Since the high-pH limit of $\Delta H'_{IMID}$ is about –6.5 kcal M^{-1} for HCA I (Figure 3), the estimated value of $\Delta H'_{IMID,A}$ will be about –15.1 kcal M^{-1}. For the cobalt enzyme a slightly smaller value of –14.6 kcal M^{-1} is computed. Table IV summarizes the thermodynamic quantities for binding of all the inhibitors, including the neutral and anionic forms of imidazole, to CA I and Co(II)CA I.

DISCUSSION

The direct determination of the enthalpy of binding of imidazole to HCA I has proven to be not feasible. This inhibitor binds relatively weakly ($K_i^{app} \approx 0.025$–0.05 M), necessitating dilution from very concentrated solutions if near saturation is to be achieved. Large, complex (biphasic), and buffer-dependent heat effects have been observed when imidazole is diluted from such solutions (data not shown). These effects swamp the heat of reaction arising from binding to the enzyme. We have consequently adopted a *difference* approach in which the heat of binding of a strongly bound sulfonamide inhibitor, methazolamide (King & Maren, 1974), is measured when it displaces a weakly bound inhibitor, present at nearly saturating concentrations, from the enzyme. Negligible heats of dilutions occur with this sulfonamide, since the titrating solution contains the sulfonamide at millimolar concentrations and the weak inhibitor is present in identical concentrations in both the enzyme and methazolamide solutions. As a central part of the analysis, it was imperative to accurately determine the intrinsic enthalpy of binding of methazolamide and to verify its pH independence, since possible pH-dependent enthalpies were being sought in the case of imidazole. The measurement of the enthalpy of binding of methazolamide over a wide pH range (Figure 2A) has thus provided an excellent means of assessing the accuracy of our studies and the validity of our approach.

Our results on CA I can also be compared with those of Binford et al. (1974), who studied methazolamide binding to bovine CA II, the high-activity isozyme, at a single pH of 8.2. Since these workers used as a reference reaction the binding of neutral methazolamide to "basic" enzyme (EOH), we have converted their results to the model of Scheme I and have computed a $\Delta H'_M$ of –12.2 kcal M^{-1}. However, they utilized in their calculations a methazolamide ionization enthalpy of 5.40 kcal M^{-1} that they obtained by extrapolation of data on benzensulfonamides, using the assumption of linear correlation between pK_s and $\Delta H'_{ion}$. Our experimentally determined value of 7.0 kcal M^{-1} for $\Delta H'_{ion}$ (Table I) is significantly different. If we utilize our value for $\Delta H'_{ion}$ and their experimental heat of reaction, we compute a $\Delta H'_M$ value of –10.8 kcal M^{-1} for binding of methazolamide to bovine CA II. This is very similar though not identical to our value of –13.5 ± 1.1 kcal M^{-1} for HCA I. Methazolamide apparently has very similar binding interactions with the two active sites.

Although no crystallographic studies have been carried out on methazolamide binding to CA isozymes, a recent high-resolution study has described the binding of the closely related acetazolamide (Diamox) to HCA II (Vidgren et al., 1990). Relevant amino acid substitutions in the active site that are in van der Waals contact with acetazolamide are the replacement of Ala-121 and Phe-91 in CA I with Val-121 and Ile-91 near the thiadiazole sulfur and His-200 with Thr-200 near the thiadiazole nitrogens. The same binding mode should occur for methazolamide in the two isozymes. Molecular mechanics calculations suggest that the van der Waals interactions of residue 121 with the aromatic ring of sulfonamides are not very strong (Vedani & Dunitz, 1985; Vedani et al., 1989; Menziani et al., 1989; Menziani & Benedetti, 1991). Future inhibitor thermodynamic studies on point mutations within a single isozyme may be most useful for testing such calculations.

The most unexpected observation of our study is, of course, the apparently small pH dependence of the enthalpy of binding of imidazole. This finding is quite unexpected, since the spectroscopic data on the imidazole complex of Co(II)HCA I, showing a large intensification at high pH (Bauer et al., 1977; Alberti et al., 1981), imply a large change in coordination around the metal ion. Recent paramagnetic proton NMR investigations have also detected changes that more or less also correlate with this transition (Luchinat et al., 1990; Bertini et al., 1991). Our results thus seem, at face value, to necessitate a reexamination of the previous conclusions that the imidazole complex of Co(II)CA I undergoes a major change from five-coordinate (or very distorted four-coordinate) at low pH to tetrahedral at high pH. However, a number of possibilities could account for the small observed calorimetric pH changes.

Luchinat et al. (1990) have recently pointed out that the presence of two distinct complexes with imidazole has been demonstrated for Co(II)HCA I and not for the native zinc enzyme. Differences in inhibitor binding to HCA I and Co(II)HCA I have been detected on occasion, such as in binding of iodide at low pH (Whitney & Brandt, 1976). Other metal ion substitutions in CA lead to larger differences in inhibitor binding [cf. Khalifah and Morley (1980)]. In order to examine this factor, our imidazole calorimetric studies were carried out on both CA I and Co(II)CA I. We find (Figure 3B) that the intrinsic enthalpy of binding of imidazole to the cobalt enzyme shows only slightly more pH dependence than seen with CA I. Furthermore, there is virtual identity between the two enzymes in the average standard enthalpy of imidazole binding over the pH range studied, the average values being

−6.1 ± 0.8 and −5.8 ± 1.1 kcal M^{-1} for the zinc and cobalt enzymes, respectively. We can safely conclude that the metal substitution had little effect on imidazole binding and its pH dependence.

It is also quite possible that compensating heat changes occurred that masked a structural change. Inspection of eqs 1–12 reveals that all protonation and deprotonation steps involving enzyme or inhibitors are "opposed" by buffer deprotonations and protonations, respectively. Thus only *differences* in heats of protonation of donor and acceptor are reflected in the reaction enthalpy. For example, in the early hypothesis that the spectral changes are due to the ionization of an adjacent, nondisplaced water ligand of the metal in the imidazole complex,

$$\geq M^{H_2O}_{Imid} \rightleftharpoons \geq M^{OH^-}_{Imid} + H^+$$

an accompanying buffer protonation step will necessarily occur: $B + H^+ = BH^+$. A change of less than 1 kcal M^{-1} in the apparent binding enthalpy would then be expected, since the metal–water enthalpy of ionization is about 9 kcal M^{-1} (Table I), a value that is similar to that of the Bis-tris propane buffer. In principle, another buffer of different ionization enthalpy may be employed to resolve this ambiguity [cf. Eftink and Biltonen (1980)]. However, this is not necessarily feasible in practice. For example, the buffering capacity in the imidazole experiments has substantial contributions from the imidazole itself, which is present at high concentrations. More seriously, many buffers with very different ionization enthalpies, such as carboxylates, have to be avoided since they are good inhibitors of carbonic anhydrase.

In the alternate view of imidazole binding one also faces a similar question: does a bound imidazole inhibitor in CA I deprotonate to yield a bound imidazolate anion and result in an enthalpy change that is masked? We earlier computed (Table IV) the intrinsic enthalpy of binding of imidazole as *either* the neutral species (eq 4) *or* the imidazolate anion (eq 11). If a pH-dependent equilibrium did exist between these two complexes, then eq 4 that was used for the computed enthalpy of binding of imidazole as the neutral species would refer to an *apparent* enthalpy $\Delta H'_{IMID,app}$ that is the pH-weighted average of the *experimental* contributions to the reaction enthalpies from binding in the two forms (as given in the relations of eqs 4 and 11):

$$\Delta H'_{IMID,app} = f^*(\Delta H'_{IMID})_{lowpH} + (1-f^*)[\Delta H'_{IMID,A} - (\Delta H'_{buf,i} - \Delta H'_{imid,2})] \quad (13)$$

Here f^* corresponds to the fraction of the complex where neutral imidazole occurs and $(1-f^*)$ refers to the fraction of the complex where the imidazole anion occurs. A pH inflection in a plot such as that of Figure 4 will obviously occur whenever the terms multiplying f^* and $(1-f^*)$ in eq 13 are *not* equal to each other within the experimental errors of about 1–2 kcal M^{-1} or so. Our observation of a near-negligible pH dependence implies that the following near equality is occurring under our experimental conditions:

$$(\Delta H'_{IMID})_{lowpH} \approx \Delta H'_{IMID,A} - (\Delta H'_{buf,i} - \Delta H'_{imid,2}) \quad (14)$$

The obligatory buffer ionization term in eq 14 provides, in principle, a clear experimental test for the existence of such a masked or attenuated ionization term in the observed enthalpy of binding. This can be done through choosing a different buffering system with a widely different (more than about 3 kcal M^{-1}) effective ionization enthalpy [cf. Eftink and Biltonen (1980)]. As noted above, an alternate buffer with a widely different enthalpy of ionization must also be shown to be noninhibitory for carbonic anhydrase. It should be emphasized that, in any case, such a demonstration will not resolve the original question as to whether the ionization belongs to the active-site group or to the bound imidazole within the complex. It will only demonstrate more clearly the existence of such an ionization in the apparent enthalpy.

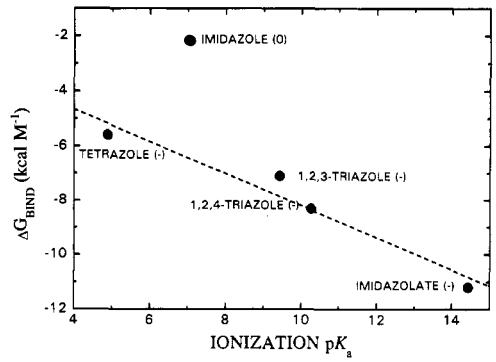

FIGURE 4: Correlation between the free energy of binding of different five-membered heterocyclic inhibitors of HCA I with the basicity of the compounds. The binding affinities of imidazole and imidazolate are taken from Khalifah et al. (1987), while those of the other compounds are taken from an analysis (see text) of the data of Alberti et al. (1979).

In retrospect, the absence of a large pH change in the apparent enthalpy of binding of imidazole (computed as the neutral imidazole species by eq 4) may not be so surprising or unreasonable. The apparent inflection of the binding affinity (in terms of neutral imidazole) at pH 8 has been previously determined (Khalifah et al., 1987) and is of the order of 0.5 kcal M^{-1}. The corresponding enthalpy change we have measured (Figure 3) amounts to 1–3 kcal M^{-1}. The magnitude of the standard free energy difference between the binding of imidazolate anion and neutral imidazole species to CA I (Khalifah et al., 1987) is roughly 9 kcal M^{-1} (Table IV). The corresponding difference in enthalpy of binding is about 10 kcal M^{-1}. Indeed, for all the inhibitors studied the enthalpy contribution to the free energy is large and becomes especially dominant as the affinity increases (Table IV). Clearly then, it is not so surprising that the enthalpy differences follow the free energy differences. Our present data are thus consistent with coupling between free energy and enthalpy changes when considering the binding of imidazole as the neutral and anionic species to the active site of CA I.

We have carried out the calorimetric studies on the binding of some imidazole analogues in order to gain more insight into the mode of binding of imidazole. Alberti et al. (1981) have shown that 1,2,3-triazole, 1,2,4-triazole, and tetrazole can all inhibit CA I as well as CA II. Additional studies on these inhibitors (Tibell et al., 1985; Khalifah et al., 1987) have confirmed and extended these findings, although no evidence has been found for their competitiveness with the substrate CO_2 (Tibell et al., 1985). These heterocycles are considerably more acidic than imidazole (Table I). A subsequent analysis of the pH dependence of their binding indicates that, formally, they inhibit the acid form of CA by binding as the deprotonated anions in the neutral and alkaline pH range (Tibell et al., 1984; Khalifah et al., 1987). However, in the case of 1,2,4-triazole, the pH profile for inhibition indicates that it resembles imidazole in that it could also bind as the neutral species, though only below about pH 5 [Khalifah et al., 1987; see also data of Tibell et al. (1985)].

Table IV compares the thermodynamics of binding of imidazole (as both neutral and anion species) with its analogues

(as anions) to HCA I. Comparison of the enthalpy of binding of the imidazole analogues as anions ($\Delta H'_{ANION} \approx -4$ to -7 kcal M^{-1}) with the binding of neutral imidazole ($\Delta H'_{IMID} \approx -5$ kcal M^{-1}) shows that there is little difference in the computed enthalpy of binding. The free energy of binding within this series is mostly contributed by the enthalpy of binding. The entropy, however, is surprisingly variable, being substantially negative only for imidazole and imidazolate.

The free energy of binding of the *anions* does appear to correlate strongly with the basicity of the coordinated nitrogen, as can be seen in Figure 4. Binding of an imidazolate anion with an enthalpy of -15 kcal M^{-1} appears reasonable in this correlation, but the binding of neutral imidazole does not follow this correlation. Its binding is much less than expected from its basicity, suggesting that neutral imidazole may bind in a different way than the series of anionic inhibitors. This would be consistent with the hypothesis that at low pH it binds off the metal in a distorted four-coordinate complex without displacing the water ligand. At high pH, the imidazolate anion would resemble the analogues in substituting the water to form tetrahedral complexes (Bertini & Luchinat, 1983; Khalifah et al., 1987). A definitive elucidation of the surprisingly complex binding modes of imidazole to CA I is greatly needed, and it may have to await crystallographic or possibly paramagnetic NMR distance studies. A central issue, of course, is which binding mode is competitive with the substrate CO_2. Arguments have been presented to favor the low-pH complex as the competitive binding mode (Khalifah et al., 1991).

ACKNOWLEDGMENT

The authors acknowledge helpful discussions with Dr. Narinder Singh.

SUPPLEMENTARY MATERIAL AVAILABLE

One table giving the apparent molar ionization enthalpy of 0.015 M Bis-tris propane buffers at different pH values with and without the added inhibitors imidazole, 1,2,3-triazole, 1,2,4-triazole, and tetrazole, and one figure showing the visible spectrum of Co(II)HCA I in the presence of imidazole, methazolamide, and a mixture of the two (2 pages). Ordering information is given on any current masthead page.

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