METAL COORDINATION GEOMETRY AND MODE OF ACTION OF CARBONIC ANHYDRASE. EFFECT OF IMIDAZOLE ON THE SPECTRAL PROPERTIES OF Co(II) AND $^{111}\text{Cd(II)}$ HUMAN CARBONIC ANHYDRASE B

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The effect of the inhibitor imidazole on the visible absorption spectrum of Co(II) human carbonic anhydrase B and on the perturbed angular correlation spectrum of $^{111}\text{Cd(II)}$ human carbonic anhydrase B have been studied at various pH values. It is demonstrated that imidazole binds both to the $^{111}\text{Cd(II)}$ and Co(II) enzymes and perturbs their respective spectra. Furthermore, spectral titrations of Co(II) with imidazole reveals binding constants similar to those determined kinetically for both the Zn(II) and the Cd(II) enzymes. Imidazole binds to both the low and high pH forms of the enzyme, and have only a weak effect on the activity linked ionization. The perturbed angular correlation results suggest that the human carbonic anhydrase B imidazole complex is five coordinated with a hydrolytic water molecule, imidazole and from the protein part His-94, His-96 and His-119 as the ligands.

The effect of acetazolamide binding of the perturbed angular correlation spectrum suggest, that this inhibitor also forms a five coordinated complex in which the NH^- and an oxygen atom of the sulfonamide group displace one or two water molecules.

Detailed analysis of the perturbed angular correlation data for $^{111}\text{Cd(II)}$ human carbonic anhydrase B suggests that the low pH form of the enzyme is four coordinated with a water molecule as the fourth ligand, whereas the high pH and active form of the enzyme might be five coordinated with a hydroxyl ion and a water molecule as the fourth and fifth ligands, respectively.

A mechanism for CO_2 hydration is proposed in which the metal is five coordinated and functions both as a LEWIS acid, activating CO_2 through direct coordination, and by activation of a coordinated water molecule for nucleophilic attack on CO_2 .

1. INTRODUCTION

Human carbonic anhydrase is a zinc metalloenzyme which contains one tightly bound zinc ion essential for catalytic activity (for an extensive review see LINDSKOG et al. 24). In addition to the reversible hydration of CO_2 , carbonic anhydrase has been found to catalyze the hydrolysis of reactive esters such as p-nitrophenyl acetate, the hydration of aldehydes, and the hydrolysis of 2-hydroxy-5-nitro-toluenesulfonic acid sultone (13). The turnover number for CO_2 hydration is of the order 10^5 to 10^6 sec^{-1} (24) which makes carbonic anhydrase one of the fastest enzymes known.

X-ray diffraction studies of human carbonic anhydrase B and C (25, 20, 14) have established that the enzyme is almost spherical in shape with a radius of about 20 Å. The Zn(II) ion is located at the bottom of a 12–15 Å deep cavity bound to three histidine residues, His-94, His-96, His-119 and a solvent molecule in approx. tetrahedral geometry.

The essential Zn(II) ion in carbonic anhydrase can be replaced by other divalent metal ions, like Co(II), Cu(II), Mn(II), Hg(II), Ni(II), Cd(II) and Fe(II). However, only Co(II), Cd(II) and Mn(II) derivatives have been shown to exhibit hydrolytic activity (23, 19, 3). The Co(II), Cd(II) and Mn(II) carbonic anhydrases have been investigated by visible absorption spectroscopy (23), EPR (30, 10), NMR (19, 9, 26, 27, 4, 5), and recently also perturbed angular correlation of gamma rays (2, 3) in order to understand the metal coordination and the catalytical mechanism.

KHALIFAH (17) has shown that imidazole is a competitive inhibitor with respect to the hydration of CO_2 by human carbonic anhydrase B. We have therefore investigated the effect of imidazole at various pH values on the visible absorption spectrum of Co(II) human carbonic anhydrase. The effect of imidazole and the strong inhibitor acetazolamide on the nuclear quadrupole interaction of $^{111}\text{Cd(II)}$ human

carbonic anhydrase B (3) have also been studied at various pH's and temperatures using the method of perturbed angular correlation of gamma rays. This method exploits $^{111}\text{Cd(II)}$ as a spectroscopic probe of the metal geometry in metalloproteins (2, 3). The demonstration of catalytic activity of the Cd(II) enzyme above pH 8 (3) shows that $^{111}\text{Cd(II)}$ is a relevant probe for human carbonic anhydrase B. It will be demonstrated that perturbed angular correlation experiments are able to give direct information of the metal geometry. The inhibition of the catalytic activity of the Cd(II) enzyme by either imidazole or acetazolamide were also studied and correlated with the results derived from the spectral studies.

2. MATERIAL AND METHODS

p-nitrophenyl acetate was obtained from BDH Chemicals, Ltd. Poole, England, and recrystallized three times from ethanol-water before use. Imidazole and 2-hydroxy-5-nitro-toluenesulfonic acid sultone were purchased from Merck, Darmstadt, West Germany. Acetazolamide was from Lederle Research Laboratories, New York, USA. Sephadex G-25 was a product of Pharmacia, Uppsala, Sweden. All buffers used were freed of trace metal contamination by extraction with 0.01 % dithizone in carbon tetrachloride (31). Glassware and cuvettes were cleaned by soaking in 1:1 nitric and sulfuric acids, followed by rinsing in metal-free distilled water. Human carbonic anhydrase B (HCAB) was isolated from hemolyzed erythrocytes by affinity chromatography (12). The apoenzyme was prepared essentially as described by COLEMAN (7) by dialysis of the enzyme against 0.01M - 1.10-phenanthroline for 2 weeks. Completely metal-free apoenzyme was obtained by affinity chromatography (12). Stock solutions of apoenzyme (20-40 mg/ml) were obtained by ultrafiltration subsequent to dialysis at the desired pH and buffer. The Cd(II) and Co(II) carbonic anhydrase

Abbreviations: PAC, perturbed angular correlation of γ -rays; HCAB, human carbonic anhydrase B; sultone, 2-hydroxy-5-nitro-toluenesulfonic acid sultone; Caps, cyclohexylaminopropane sulfonic acid; Co(II) MesTrenBr, Cobalt(II)-tris (2-dimethylaminoethyl) -amine-bromide.

derivatives were obtained by addition of a threefold excess of the metal ions prepared from spectrographically pure sulfate salts (Johnson, Matthey Chemicals, Ltd. London, England) in metal-free distilled water. The protein concentration was determined from the absorbance at 280 nm using a molar absorptivity of $4.69 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (23). The enzymatic activity was determined routinely using *p*-nitrophenyl acetate (1mM) as a substrate by following the change in absorbance at 348 nm (1). The inhibition of the various metalcarbonic anhydrase derivatives by imidazole were studied using 2-hydroxy-5-nitrotoluenesulfonic acid sultone as the substrate (13). For both substrates the observed rates were corrected for the rate of hydrolysis in the absence of enzyme, and furthermore, the apoenzyme was used as control. All kinetic determinations were performed at 25°C. Inhibition constants were determined from Dixon plots.

Absorbance measurements and visible absorption spectra were obtained with a Cary Model 118 C spectrophotometer at 25°C. Spectral titrations of cobalt (II) carbonic anhydrase with imidazole were performed by adding in a cuvette, microliter volumes of a 2.0 M solution of imidazole in the same buffer to 1.0 ml solution of the enzyme, approx. $2 \times 10^{-4} \text{ M}$. Results were corrected for enzyme dilution due to inhibitor addition. The apparent dissociation constant K_{app} , was obtained by plotting $1/(A - A_0)_\lambda$ vs. $1/[I]$, where A_0 is the absorbance of the cobalt (II) carbonic anhydrase in the absence of imidazole (I) and A is the absorbance in its presence at wavelength λ . The intercept of the abscissa of the resulting straight line is $-1/K_{\text{app}}$.

The theory and experimental procedure for the PAC experiments, the production and isolation of $^{111}\text{Cd(II)}$ as well as the preparation of $^{111}\text{Cd(II)}$ carbonic anhydrase have been described in details (3). Before each measurement the enzyme solution was passed over a Sephadex G-25 column ($1.4 \times 15 \text{ cm}$) equilibrated in the desired buffer for the particular experiment in order to separate Cd(II) bound to the enzyme from free Cd(II) and to obtain enzyme samples free of Cl^- .

The pH of the samples were determined im-

mediately after the experiment with a Radiometer pHM 63 pH-METER equipped with a GK 2301/B combination electrode. The pH values at -196°C are defined as the values measured on the samples at 0°C .

Since the Cd(II) enzyme is much more sensitive to anion inhibitors (see section 3.2) such as Cl^- and HCO_3^- , particular precautions were taken to minimize these contaminations. Thus pH measurements were only performed on aliquots of stock solutions of buffer or sample, and all buffers were prepared immediately before use to prevent CO_2 uptake. The time spectrum from a PAC measurement between two gamma rays is represented by the following function (3)

$$W(\Theta, t) = e^{-t/\tau_n} (1 + A G(t, \Delta E_{\text{hf}}) (3/2 \cos^2 \Theta - 1/2)) \quad [1]$$

where τ_n is the lifetime of the nuclear state, A is the unperturbed amplitude, Θ is the angle between the two gamma rays, t is the time of emission of the second gamma ray with respect to the first one and $G(t, \Delta E_{\text{hf}})$ describes the perturbation from the surrounding molecular structure via the hyperfine interaction characterized by the energy splitting ΔE_{hf} . In the case of $^{111}\text{Cd(II)}$ the hyperfine splitting results entirely from the nuclear quadrupole interaction (3). This interaction is determined by the components of the electric field gradient

$V_{\alpha\beta} = (\partial/\partial\beta E_\alpha(\vec{r}))_{\vec{r}=0}$ where $\alpha, \beta = x, y$ or z and $\vec{E}(\vec{r}) = (E_x(\vec{r}), E_y(\vec{r}), E_z(\vec{r}))$ is the electric field set up by the molecular structure at the position $\vec{r}$ in a coordinate system with origin at the $^{111}\text{Cd(II)}$ nucleus. In the present experiments where the molecules were randomly oriented the factor $G(t, \Delta E_{\text{hf}})$ was determined by the following two parameters, the field gradient $|V_{zz}|$ (through the observed frequency ω_0 which is proportional to $|V_{zz}|$) and $\eta = \frac{|V_{xx} - V_{yy}|}{V_{xx} + V_{yy}}$. The spectra have been recorded in the angles 180° and 90° , and the ratio $W(180^\circ, t)/W(90^\circ, t)$ has been formed.

In order to analyse the pH behavior of the field gradient $|V_{zz}|$ and the asymmetry parameter η a semiempirical point charge model has been used for the $^{111}\text{Cd(II)}$ enzyme PAC results. The model assumes that bonding occurs only via

bonding, i.e. no π bonding is allowed. The contribution from the various σ orbitals to the field gradient $|V_{zz}|$ can then be represented by a pseudo point charge as determined experimentally (3). This model, also known as the additivity model, has attained strong support from the analysis of a large series of Mössbauer nuclear quadrupole interaction measurements on Sn(IV) complexes, which are isoelectronic with Cd(II) (28). because a single $|V_{zz}|$ value is sufficient to determine this pseudo point charge the other parameter η can be used to predict molecular properties from a single PAC experiment. In the case where n different PAC experiments are performed for the same enzyme metal complex, n different $|V_{zz}|$ and η values are generated, reflecting for example n different forms obtained by changing the pH or by adding various inhibitors. Under these conditions it is possible to use $n-1$ of the $|V_{zz}|$ values and all of the η values to deduce structural information for the metal site.

3. RESULTS

3.1 Visible absorption spectra of cobalt (II) carbonic anhydrase B

The absorption spectrum of cobalt (II) carbonic anhydrase is sensitive to pH and the binding of various inhibitors to the cobalt ion, producing characteristic spectral changes (23,24).

As shown in Figs. 1 and 2 the competitive inhibitor imidazole also perturbs the cobalt (II) HCAB spectrum. At pH 7.3 (Fig. 1) increasing contractions of imidazole progressively abolishes the 640 and 615 nm bands, while the absorbance at both 520 and 560 nm increases, with an isosbestic point at 606 nm. The spectral effects of imidazole at pH 9.5 (Fig. 2) is similar to those observed at pH 7.3 but in addition a new band at 590 nm is apparent, and the isosbestic point is located at 612 nm. The double reciprocal plots of the absorbance titration at 590 nm at various pH values are linear (Fig. 3) with similar dissociation constants, K_{app} , of approx. 50 mM.

It is apparent from Figs. 1 and 2 that in the presence of imidazole the spectrum of Co(II) HCAB is pH dependent. The pK for this

Figure 1. Effects of imidazole on the absorption spectrum of Co(II) HCAB in 0.05M-Tris-SO₄, pH 7.3, 25°C (—). Addition of 20mM (---), 39 mM (.....), 95 mM (— · —), and 333 mM (-----) imidazole. All data were corrected for dilution.

Figure 2. Effect of imidazole on the absorption spectrum of Co(II) HCAB in 0.05M-Tris-SO₄, pH 9.5, 25°C (—). Addition of 25mM (---), 39 mM (.....), 95 mM (— · —) and 333 mM (-----) imidazole. All data were corrected for dilution.

spectral pH-dependence was determined at various imidazole concentrations (Fig. 4) plotting the change in absorbance at 596 nm, the isosbestic point for the spectral pH titration of Co(II)HCAB itself (23). For the three imidazole concentrations the curves could be fitted to a single ionization with a pK_{app} of 7.9 ± 0.1 .

Figure 3. Double reciprocal plots ($1/(A-A_0)$ vs. $1/[I]$) at 590 nm for Co(II)HCAB spectral titrations with imidazole (I) at pH 7.3 (▲), pH 7.8 (●), pH 8.5 (■) and pH 9.5 (▼). The data were calculated from spectral titrations similar to those shown in Figure 1 and 2.

The pK_{app} for Co(II)HCAB without imidazole was determined from the change in absorption at 640 nm with pH to be 7.5 ± 0.2 similar to the value previously reported (24). Thus the ionization which has been suggested to control the activity of the enzyme (22) is also present when imidazole is bound to the cobalt (II) ion in the enzyme. In addition these results show that neutral imidazole binds to both the low and high pH forms of the Co(II)HCAB.

3.2 Enzyme kinetic studies

The inhibition of the Zn(II) enzyme at pH 8.0 and 9.5 by imidazole using the sultone as substrate showed very similar K_i values of approx. 20mM (Table I), identical to the values obtained by KHALIFAH (17) using CO_2 as substrate. The K_i values for inhibition of the Cd(II) enzyme by imidazole at pH 8.5 and 9.5

Figure 4. The effect of pH on the absorbance at 596 nm of Co(II)HCAB in the presence of 333 mM (●, —) 182 mM (▼, ---) and 95 mM (■, ····) imidazole. The data were calculated from spectral absorbance titrations similar to those shown in Figures 1 and 2. the curves represent least square fits to a single ionization with a pK of 7.9.

Figure 5. Dixon plots of the imidazole (I) inhibition of Cd(II)HCAB hydrolysis of the sultone substrate in 0.05 M Tris- SO_4 , pH 8.5 (●) and pH 9.5 (▲). The pseudo first order rate constant, k_{enz} , was determined as the average value obtained at two sultone concentrations 50 and 75 μM .

Table I

Inhibition constants for Zn(II)HCAB and Cd(II)HCAB for the inhibitors imidazole, acetazolamide and Cl^-

Metal derivatives	pH	Inhibitor	K_i value in M
Zn(II)HCAB ^{a)}	8.0	imidazole	16×10^{-3}
"	9.5	"	20×10^{-3}
Cd(II)HCAB ^{a)}	8.5	"	30×10^{-3}
"	9.5	"	14×10^{-3}
Zn(II)HCAB ^{b)}	8.0	acetazolamide	0.59×10^{-6}
Cd(II)HCAB	8.5	"	18×10^{-6}
"	9.4	"	37×10^{-6}
Zn(II)HCAB ^{b)}	7.5	Cl^-	11×10^{-3}
Cd(II)HCAB ^{b)}	9.5	"	0.28×10^{-3}

^{a)} derived from Dixon plots using sultone as substrate^{b)} derived from Dixon plots using p-nitrophenyl acetate as substrate

were 30 and 14 mM, respectively (Fig. 5 and Table I). Since we have previously shown that the activity linked pK for the Cd(II) enzyme is 9.1 ± 0.2 in contrast to 7.3 for the Zn(II) enzyme (3), the results suggest that the neutral imidazole binds both to the low and high pH form of the Cd(II) enzyme.

Acetazolamide was found to inhibit the Zn(II)HCAB at pH 8.0 with a K_i of $6 \times 10^{-7}\text{M}$, similar to the value previously obtained by S. LINDSKOG (personal communication). The Cd(II)HCAB was also inhibited by acetazolamide with a K_i of $1.8 \times 10^{-5}\text{M}$ and $3.7 \times 10^{-5}\text{M}$ at pH 8.5 and 9.4 respectively

(Table I). Finally, it was found that Cl^- inhibits the Cd(II) enzyme much stronger ($K_i = 0.28\text{ mM}$) than the Zn(II) enzyme ($K_i = 11\text{ mM}$) (Table I).

3.3 PAC spectra

Some of the PAC spectra are presented in Fig. 6 and the nuclear quadrupole interaction parameters derived from least square fittings to the PAC spectra are presented in Table II.

From Table II it is seen that at -196° two different forms (ω_{01} , η_1 , and ω_{02} , η_2) of the Cd(II) enzyme exist in the presence of imidazole, both being different from the high and low pH forms in the absence of imidazole. The corresponding

Table II

Nuclear quadrupole interaction parameters for $^{111}\text{Cd(II)-HCAB}$ in the presence of various inhibitors

Inhibitor	Temp ($^\circ\text{C}$)	pH	$\omega_{01}(\text{Mhz})$	η_1	$\omega_{02}(\text{Mhz})$	η_2	% ω_{02}
0.2M-Imidazole	-196	9.0	114 ± 11	0.10 ± 0.27	157 ± 14	0.16 ± 0.20	52 ± 18
0.2M-Imidazole	-196	10.0	106 ± 20	0.37 ± 0.29	168 ± 16	0.46 ± 0.12	64 ± 19
95mM Imidazole	22	7.8	124 ± 12	0.30 ± 0.23	(61)	(0.9)	16 ± 22
1mM-Acetazolamide	-196	9.5	120 ± 17	0.35 ± 0.24	180 ± 23	0.36 ± 0.29	43 ± 24
0.8mM-Acetazolamide	22	7.9	106 ± 14	0.44 ± 0.16	(230)	(0.1)	18 ± 23
No inhibitor present	22	7.9	79 ± 8	0.73 ± 0.26	213 ± 33	0.00 ± 0.41	35 ± 19
0.5M-Chloride	22	7.7	97 ± 5	0.72 ± 0.16	(65)	(1.0)	3 ± 20

At -196°C the buffer is 0.05 M-Tris and at 22°C 0.027 M-Tris- SO_4 . A 50.8% sucrose solution has been used at 22°C . The enzyme concentration was between 10 – 100 μM .

Figure 6. PAC time spectra ($W(180^\circ)/W(90^\circ)$) for $^{111}\text{Cd(II)HCAB}$. A: in the presence of 95 mM imidazole and 0.027 M-Tris- SO_4 , 50.8% (weight) sucrose pH 7.8, 22°C . B: in the presence of 200 mM imidazole and 0.05 M -Caps, pH 10.0, -196°C .

theoretical G factors (eqn. [1]) are shown in Fig. 7 together with those from the low and high pH form of Cd(II)HCAB without inhibitor. At 22°C and pH 7.8 in the presence of imidazole essentially one frequency existed which appeared to be identical with the low frequency form present at pH 9 and 10 at

Figure 7. The time dependence of the perturbation factor G in: A for the low (—) and high (----) pH forms of $^{111}\text{Cd(II)HCAB}$, and in B: for the low (—) and high (----) pH forms of the $^{111}\text{Cd(II)HCAB}$ imidazole complex.

-196°C. The imidazole PAC results suggest that imidazole binds both to the low and high pH form of the Cd(II) enzyme. On this basis analysis of the low and high pH form of the nuclear quadrupole interaction parameters ω_0 and η with and without imidazole bound to Cd(II)HCAB were performed using the parameters in Table III. These four species of Cd(II)HCAB produced a total of 4 ω_0 values and 4 η values thereby leaving 7 informative parameters as explained in Section 2. The analysis was based on the assumption that the metal ligands His-94, His-96 and His-119 were binding in tetrahedral angles with equal strength to the metal ion and that the imidazole bond was situated opposite to the His-96 metal bond (Fig. 8) Based on this model various possibilities for four or five metal ligands were investigated. As the fourth ligand (S_4 in Fig. 8) was tested either a water molecule, a hydroxyl group or no ligand at all. For the low and the high pH form of Cd(II)HCAB without the presence of imidazole it was examined whether a water molecule could be placed at the imidazole binding site (S_5 in Fig. 8) either binding to the low pH form, to the high pH form or to both the forms of Cd(II)HCAB under the assumption that the fourth ligand site was occupied by a water molecule for the low pH form and a hydroxyl ion for the high pH form. Finally, the possibility was considered that one of the metal histidine bonds was broken at low pH. The parameters obtained by a least square fit procedure were the field gradient strength of the fourth ligand S_4 , with or without imidazole at the fifth ligand site, the polar angles Θ_4 , Φ_4 of the fourth ligand and the field gradient strength of the imidazole bond S_5 (Fig. 8). It should be

Figure 8. Diagram of the geometry model used in the analysis of the PAC data for $^{111}\text{Cd(II)HCAB}$ and for its imidazole complex. N_{96} , N_{94} and N_{119} are the metal binding nitrogen atoms of His-96, His-94 and His-119, respectively having an angle of 109.5° between any two of them. S_4 represents the solvent metal ligand site with the polar angles Θ_4 and Φ_4 . S_5 is the imidazole site located in opposite direction to the metal- N_{96} .

Table III

Summarized quadrupole interaction parameters at -196°C

$^{111}\text{Cd(II)HCAB}^a)$		$^{111}\text{Cd(II)HCAB}$ imidazole complex ^{b)}	
low pH form $\omega_0 = 95 \pm 4 \text{ Mhz}$ $\eta = 0.74 \pm 0.10$	high pH form $\omega_0 = 244 \pm 18 \text{ Mhz}$ $\eta = 0.17 \pm 0.16$	low pH form $\omega_0 = 112 \pm 10 \text{ Mhz}$ $\eta = 0.23 \pm 0.20$	high pH form $\omega_0 = 162 \pm 11 \text{ Mhz}$ $\eta = 0.38 \pm 0.10$

a) taken from ref. 3.

b) weighted mean values for the two imidazole experiments at -196°C (table II)

Figure 9. A two-dimensional plot of the probability of exceeding the least square minimum value obtained in the PAC analysis of the $^{111}\text{Cd(II)HCAB}$ data for a water molecule at the imidazole site S_5 (see Fig. 8) with a field gradient strength, relative to the histidine ligands of S_5^L were L denotes the low pH form with the S_4 site occupied by a water molecule, and of S_5^H where H denotes the high pH form with the S_4 site occupied by a hydroxyl ion.

noted that the change of a water ligand to a hydroxyl ligand was described by adding a negative point charge in 2 Å distance from the metal center (3).

Various results for different field gradient strengths of a water ligand at the imidazole site are plotted in a two dimensional probability map in Fig. 9. This map shows that the imidazole site in the absence of bound imidazole might be occupied by a water molecule in the high pH form S_5^H (where the fourth ligand is assumed to be a hydroxyl ion), but not, or only with a very weak binding, in the low pH form S_5^L (where the fourth ligand is assumed to be a water molecule). In the acceptable cases where $S_5^L = 0$ (Fig. 9) (i.e. the enzyme metal complex is four coordinated at low pH and either four coordinated $S_5^H = 0$ or five coordinated $S_5^H = 0$ at high pH) the field gradient strength of the imidazole-Cd(II) bond varies between 0.3–0.7 relative to the field gradient strength of the histidine-Cd(II) bonds and the field gradient strength and Θ_4, Φ_4 of the hydrolytic water

molecule Cd(II) bond is approximately the same as that for the histidine Cd(II) bonds and close to $90^\circ, 240^\circ$, respectively.

Four other possibilities investigated without any water molecule bound to the fifth coordination site S_5 were as follows:

- 1) Cd(II) is coordinated to a water molecule at the S_4 site in the low pH form, but not in the high pH form.
- 2) Cd(II) binds a water molecule at the S_4 site in the low pH form.
- 3) Cd(II) binds a water molecule at the S_4 site both to the low and the high pH form, but one of the Cd histidine bonds is broken for the low pH form.
- 4) Cd(II) binds no water molecule at any pH but one of the Cd(II) histidine bonds is broken for the low pH form.

In the situations 1-4 we get the result that $P < 1\%$ where P is the probability of exceeding the least square minimum value.

Finally, the validity of the assumption that imidazole binds opposite to the Zn(II)-N₉ (Fig. 8) bond was partly investigated by searching the least square in the angles $\Theta_{\text{imidazole}}$ and $\Phi_{\text{imidazole}}$ whereby no probability P above 1% was found for $160^\circ > \Theta_{\text{imidazole}} > 20^\circ$.

In summary, the analysis showed that it was necessary to include a hydrolytic water molecule as a metal ligand in order to have an acceptable least square minimum, whereas alternatives such as the addition of a water molecule as a function of pH or protonation of one of the metal bound histidine residues at low pH were unacceptable. These conclusions were also reached in our previous work (3) based on a broad general discussion of the pH dependent change of the nuclear quadrupole interaction parameters ω_0 and η . In addition, the finding that it was not possible to have the fifth ligand position occupied by a water molecule *both* in the low and in the high pH form of $^{111}\text{Cd(II)HCAB}$ seems reasonable, since in this case the nuclear quadrupole interaction parameters in the presence and absence of imidazole would have been very similar.

A concentration of 10^{-3}M -acetazolamide at -196°C at pH 9.5 produced a new PAC spectrum relative to the uninhibited Cd(II)HCAB as can be seen in Table II. Two forms of the acetazolamide complex seems to be present, which within the limit of uncertainty cannot be separated from the two Cd(II)HCAB imidazole

forms. The nuclear quadrupole interaction parameter at pH 7.9 and 22°C in the presence and absence of acetazolamide are seen to be clearly distinguishable whereas the parameters for the two acetazolamide experiments at pH 7.9, 22°C and pH 9.5, -196°C might be identical.

The presence of 0.5M-Cl⁻ at pH 7.7, 22°C also changed the nuclear quadrupole interaction parameters significantly relative to the parameters derived in the absence of any inhibitor at pH 7.9, 22°C (Table II). Our previous studies of the effect of 20mM Cl⁻ at pH 5.6, 22°C and 0°C on the PAC spectrum did not reveal any spectral perturbations (3). Whether this discrepancy is due to the rather large uncertainty, or a much weaker binding of Cl⁻ at pH 5.6 than at pH 7.7 or due to a change in the nuclear quadrupole interaction parameter for either Cd(II)HCAB or for the Cl⁻-Cd(II)HCAB complex are not possible to evaluate with the present data. It should be noted that the lowest frequency determined at pH 7.9, 22°C (the present work), the lowest frequency determined at pH 9.1, 22°C (3), and the dominating frequency at pH 5.6, 22°C (3) all without the presence of inhibitors, might be identical within the uncertainty.

3.4 Calculation of the metal geometry from X-ray data

The angles for the Zn(II) ligands in HCAB have been calculated using the coordinates for the Zn(II) ion, the three histidine ligand nitrogens, the center of the solvent ligand, the atom closest to Zn(II) for imidazole and the two

ligand atoms in acetazolamide (kindly provided by K. K. KANNAN). The Zn(II) HCAB ligands are not significantly different when either imidazole or acetazolamide is bound to Zn(II)HCAB. The results can be seen in Table IV. The uncertainty is estimated to be $\pm 15^\circ$.

All binding distances for Zn(II)HCAB are approx. 2 Å, except for the imidazole metal bond and one of the two metal bonds for acetazolamide where the distance is approx. 2.7 Å (15, 16).

4. DISCUSSION

The X-ray diffraction data of Zn(II) human carbonic anhydrase B have shown that the high pH form of Zn(II)HCAB is four coordinated with His-96, His-94 and His-119 and a solvent molecule as ligands (14). However, it is not known whether the solvent ligand is a water molecule or a hydroxyl ion. Furthermore, there is no information about the metal coordination geometry for the low pH form. In addition there is still no direct knowledge about the CO₂ binding site in carbonic anhydrase.

We have previously established that the method of PAC applied to ¹¹¹Cd(II)HCAB is able to give spectroscopic information about the catalytical important metal site (2, 3). It was further suggested that the most probable explanation of the pH dependent change of the nuclear quadrupole interaction for ¹¹¹Cd(II)HCAB was a titration of a metal bound hydrolytic water molecule (3).

Table IV

Ligand geometry for Zn-HCAB deduced from X-ray data^{a)}

Angle (in degrees)	His ₉₆	His ₁₁₉	His ₉₄	Solvent ligand	Imidazole ligand	Acetazolamide	
						ligand 1	ligand 2
Θ	0	88	126	99	145	99	154
φ	0	0	115	225	279	197	222

a) The coordinate system chosen has a Z axis along the His₉₆-Zn bond and X-Z plane in the His₉₆-Zn - His₁₁₉ plane and origin at the Zn center, Θ and φ are polar angles.

4.1 Imidazole binding

KHALIFAH (17) has shown that neutral imidazole is a competitive inhibitor of CO_2 hydration for Zn(II)HCAB . The present kinetic measurements on Zn(II)HCAB and Cd(II)HCAB as well as the visible absorption data on Co(II)HCAB do all favour the neutral imidazole molecule as the inhibitory species. The visible absorption spectra of Co(II)HCAB (Figs. 1 and 2) and the PAC spectra of $^{111}\text{Cd(II)HCAB}$ (Fig. 6) in the presence of imidazole suggest the presence of two characteristic forms of the enzyme imidazole complex, a low pH form and a high pH form. Thus it appears that imidazole binds both to the low and the high pH form of HCAB and that the activity linked ionization is maintained also in the imidazole-enzyme complex. Furthermore, there is no major difference between the K_1 values for the Zn(II) and Cd(II) enzyme and K_{app} values for the Co(II) enzyme. This observation is particularly remarkable for the Cd(II) enzyme considering the large shift in the activity pK for the Cd(II) enzyme ($\text{pK} = 9.1$) versus the Zn(II) enzyme ($\text{pK} = 7.3$). It is therefore strongly indicated that the binding site of imidazole is different from the activity linked ionization site.

Several important points emerge from the analysis of the PAC data with and without imidazole bound to Cd(II)HCAB . It is demonstrated that among the various theoretical models tested (Section 3.3) only those which include a hydrolytic water molecule as a metal ligand are acceptable. Furthermore, the diagram in Fig. 9 shows that without imidazole a second metal bound water molecule might be present, but only in the case of the high pH form of Cd(II)HCAB . If these results for the Cd(II) enzyme also pertain for both the Zn(II) and Co(II) enzyme, they suggest that the low pH and hydrolytic inactive form of the enzyme is four coordinated with a water molecule as the fourth ligand. In contrast, the high pH and active form of the enzyme might be five coordinated with a hydroxyl ion, and a water molecule as the fourth and fifth ligands respectively. Hence, the large change in the absorption spectrum with pH seen for the Co(II) -carbonic anhydrase (24) would seem to reflect a change in coordination geometry con-

comitantly with induction of enzymatic activity. Based on spectroscopic data DENNARD and WILLIAMS (8) have previously suggested the presence of both four and five coordinated metal geometries in carbonic anhydrase. Furthermore recent magnetic circular dichroism studies on bovine Co(II) carbonic anhydrase (11) also suggest that the low pH form of the enzyme is four coordinated, while the high pH form is five coordinated.

Detailed analysis of the PAC data of the $^{111}\text{Cd(II)HCAB}$ -imidazole complex showed that the position of the ligand atom in imidazole has to be located approximately opposite to the His-96-Cd(II) bond. Furthermore, the configuration of the imidazole enzyme complex (Fig. 10 A) is close to the five coordinated geometry of a trigonal bipyramid, which in this case would have an angle of 109.5° to the axial symmetry direction ($\text{N}_{96}\text{-Cd-N}$ (of imidazole)) except for the hydrolytic water ligand with an angle of approx. 90° . Such a configuration is close to the geometry observed for the five coordinated Co(II)MesTrenBr complex (6). Recently the three-dimensional crystal structure of the Zn(II)HCAB -imidazole complex has been reported (15), revealing in accordance with the present data, that imidazole binds to a fifth coordination site without displacing the solvent molecule at the fourth coordination position (15). Furthermore the binding angles for the hydrolytic water ligand and the imidazole ligand atom ($\Theta = 180^\circ$) to Cd(II) in Cd(II)HCAB deduced from the PAC data are seen to be close to the angles calculated from the X-ray diffraction data (Table IV). The effect of imidazole on the absorption spectrum of Co(II)HCAB , on the PAC spectrum of $^{111}\text{Cd(II)HCAB}$, on the X-ray diffraction of Zn(II)HCAB crystals (15) and on the EPR spectra of Co(II) bovine carbonic anhydrase (10) all demonstrate that imidazole binds directly to the metal. Thus it would seem that, if the high pH form of the enzyme is five coordinated, imidazole binds to the metal by displacing a water molecule, whereas imidazole binds to the low pH-form (four coordinated) of the enzyme at an open coordination site.

4.2 Acetazolamide binding

Aromatic and heteroaromatic sulfonamides are extremely effective inhibitors of the catalytic activity of carbonic anhydrase with K_i values from 10^{-3} to 10^{-9} M (24). The visible absorption spectrum of the Co(II) enzyme is perturbed when a 1:1 complex of the Co(II) enzyme and acetazolamide is formed (11). The enzyme bound acetazolamide spectrum is pH independent (11), but appears to be almost identical to the spectrum of the high pH form of the Co(II) enzyme saturated with imidazole (Fig. 2). Furthermore the two forms identified in the PAC spectrum of Cd(II)HCAB in the presence of acetazolamide appears similar to the two imidazole Cd(II)HCAB forms (Table II), suggesting that the metal geometry is similar in the presence of these two inhibitors.

The kinetic investigations performed by TAYLOR (29) and LINDSKOG (21) showed that only the two equilibria described in eqns. 2 and 3 can explain the pH dependence of sulfonamide inhibition.

where EH and E^- denote the low and high pH form of carbonic anhydrase, respectively, and AH and A^- the neutral and ionized forms of the sulfonamide group, respectively. For acetazolamide which is studied in the present report the pH dependence of the K_i values for eqn. 2 and eqn. 3 can be written (21)

$$K_i^{[2]} = (1 + K_E/[\text{H}^+]) F^{[2]} \quad [4]$$

$$K_i^{[3]} = K_S(1 + K_E/[\text{H}^+]) F^{[3]}/K_E \quad [5]$$

where K_E is the dissociation constant for the activity linked group on the enzyme, and K_S the dissociation for the sulfonamide group and where $F^{[2]}$ and $F^{[3]}$ only depends on the intrinsic

binding of acetazolamide to the enzyme. The ratio between the K_i values of Cd(II)HCAB and Zn(II)HCAB at pH 8.5 is approx. 11 (from Table I and using a K_i value of 1.6M for Zn(II)HCAB (S. LINDSKOG, personal communication)) which can be compared with the ratio calculated from [4] and [5] equal to approx. 7×10^{-2} and 5, respectively, neglecting any change in $F^{[2]}$ and $F^{[3]}$. Thus combination of the neutral acetazolamide with the basic form of the enzyme seems to be favoured if the intrinsic binding for Zn(II)HCAB and Cd(II)HCAB is identical. It has been proposed that sulfonamides are bound in the anionic form (18) and it is therefore necessary to include an additional step for eqn. 3 such as

or if we assume that sulfonamide displace either H_2O or OH^- we would get the following modifications of eqns. 2 and 3

For eqn. 3' we should note that also the following equation must be considered

which however do not agree with the pH dependency of sulfonamide inhibition (21, 29), unless the second order rate constant for the reaction to the right in eqn. 7 is orders of magnitudes less than the corresponding rate for the reaction to the right in eqn. 3'. A molecular proposal for the equilibrium in eqn. 6 and two configurations for EA^- species are shown in Figs. 10 B and C, respectively. In both cases we have a five coordinated metal geometry and either a neutral or a negatively charged ligand at the fourth coordination position, which

Figure 10. Proposals for the metal geometry in HCAB. A: Metal geometries for the low (right) and high (left) pH forms of the imidazole-HCAB complex. B: Metal geometries for acetazolamide bound to the low (right) and high (left) pH forms of HCAB. C: Two configurations for acetazolamide bound to HCAB, when the solvent ligand is displaced. D: Metal geometries for the low (left) and high (right) pH forms of HCAB. E: Binding and proposed hydrolysis of CO_2 to metal bound HCO_3^- .

correlate well with the similarity between the two imidazole and the two acetazolamide forms identified by PAC. This similarity of the coordination geometry might also explain the nearly identical absorption spectra for the high pH form of the Co(II)HCAB-imidazole complex and the Co(II)HCAB-acetazolamide complex. A five coordinated geometry has also been observed by X-ray diffraction for the acetazolamide enzyme complex (16). Furthermore, the bond angles obtained from the X-ray data (Table IV) for the two acetazolamide metal ligands are, respectively, very close to the imidazole metal ligand angles and the solvent metal ligand angles. For the metal acetazolamide geometries shown in Figs. 10 B and C only scheme C is not in conflict with the X-ray diffraction results, because no hydrolytic water molecule can be placed between the acetazolamide metal ligand and the metal (16). However, scheme B could represent intermediate states before one or both the configurations sketched in scheme C is reached by displacing the water molecule if eqn. 6 represents intermediate steps of eqn. 3'.

4.3 Metal geometry and mechanism

From the present work and from the X-ray crystallographic studies (15, 16) it is established that the metal in carbonic anhydrase has the capacity of five coordination. The suggestion that the activity linked ionization group is a metal coordinated hydrolytic water molecule (22) is further supported by the present results of the detailed analysis of the PAC data. Furthermore, it is demonstrated that imidazole binds both to the low and high pH form of Co(II)HCAB and Cd(II)HCAB. From NMR studies on the Mn and Co enzymes (19, 9) it has been suggested that the pH dependence of the activity arises from a titration of a metal bound water molecule such that the water metal bond is broken at low pH. Such a proposal is not in conflict with the present data if this water molecule becomes a ligand at high pH at the fifth coordination site.

Based on the finding that imidazole is a competitive inhibitor of CO₂ hydration (17), and the present results, that imidazole binds to a fifth metal coordination site, we propose that

CO₂ also binds to this site. This suggestion has important bearings on the mechanism of carbonic anhydrase, since in this case the metal can act both as a Lewis acid, activating CO₂ through direct coordination (Fig. 10 E), and by activation of a coordinated water molecule (Fig. 10 D) for nucleophilic attack on CO₂ (Fig. 10 E). Based on the X-ray crystallographic studies on the HCAB imidazole complex KANNAN et al. (15) have recently proposed a similar mechanism for CO₂ hydration.

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