^{13}C and ^{1}H NMR studies of imidazole binding to native and Co(II)-substituted human carbonic anhydrase I

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Abstract

The inhibition constant of imidazole toward human carbonic anhydrase I has been directly determined at pH 6.8 and 8.8 through ^{13}C NMR. The data are in agreement with those obtained by indirect methods and confirm that the binding affinity of imidazole is substantially constant in the pH range investigated. Analysis of the isotropically-shifted signals in the ^{1}H NMR spectra of the Co(II)-substituted enzyme interacting with imidazole at high and low pH in the presence of sulfate as counterion indicates the likely formation of a five-coordinate HCAI–imidazole–sulfate adduct at low pH. The binding mode of imidazole in the active site of the enzyme is discussed.

Introduction**

Among the wide variety of inhibitors of the low activity carbonic anhydrase isoenzyme I, imidazole is the only one that has been hitherto found to be competitive with carbon dioxide [1]. The affinity of imidazole for the native enzyme appears to be essentially independent of pH in the pH range 7–9 [2]: hence, the binding seems not to be modulated by the ionization of the active site, unlike other inhibitors of this enzyme [3]. Imidazole binding to the Co(II)-substituted enzyme studied through electronic spectroscopy is found slightly pH-dependent with an apparent pK_a of about 8 [4]; however such a pH effect has not yet been unambiguously related to the binding mode of this inhibitor.

In this paper we have studied the interaction of imidazole with the native enzyme through inhibition measurements of the $CO_2 \rightleftharpoons HCO_3^-$ interconversion at chemical equilibrium: in particular, we have evaluated at different pH values the effect of imidazole on the rate of the HCAI-catalyzed CO_2/HCO_3^- exchange measured from the ^{13}C NMR linewidth of $^{13}CO_2$ and $H^{13}CO_3^-$. This technique allowed direct determination of the imidazole inhibition constants, which have been compared with those so far obtained with indirect methods [2] and from steady state measurements [1]. Furthermore, the ^{1}H NMR spectra of the CoHCAI–imidazole adduct have been recorded at different pH values with the aim of gaining information about the inhibitor binding mode.

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**Abbreviations used throughout: CA = carbonic anhydrase; HCAI = human carbonic anhydrase (isoenzyme I), NMR = Nuclear Magnetic Resonance, MES = 2-(*N*-morpholino)-ethanesulfonic acid.

Experimental

Samples for ^{13}C NMR measurements

Samples of native HCAI at pH 6.8 were prepared as follows. A 10 mm O.D. cap screw NMR tube equipped with a self-sealing chromatography teflon set was used, and all chemicals were introduced with a microsyringe. Amounts of 0.5 M H_2SO_4 were added to D_2O initial solutions containing different concentrations of ^{13}C-enriched bicarbonate. The ^{13}C-enriched CO_2 is thus produced *in situ* in the sealed tube and equilibrates between the liquid and the gaseous phase. The concentration of bicarbonate and carbon

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dioxide, and consequently the pH of the solution, were calculated from the area of the signals after an accurate standardization procedure. After obtaining pH 6.8, the ionic strength was adjusted to 0.2 M with concentrated Na_2SO_4. Enzyme (unbuffered solution) was then added. $H^{13}CO_3^-$ and enzyme concentrations were in the range 12.5–51 mM and 10–30 μM, respectively. To each sample at different initial HCO_3^- concentration increasing amounts of a 1 M imidazole–SO_4^{2-} aqueous solution at pH 6.8 were added to give final imidazole concentrations of 15, 60 and 120 mM. Due to the sulfate content of the inhibitor solution the ionic strength of the samples is not rigorously constant, but the final value of about 0.26 at the highest imidazole concentration does not significantly affect the data, as well as the corresponding sulfate concentration (70 mM) [5]. The constancy of the pH in all the experiments was checked by adding acetazolamide to the last sample of each series: the pH variation has never exceeded 0.1 units.

Measurements at pH 8.8 were performed with the same equipment: now the pH was adjusted with NaOH and initial and final values were checked by a pH meter. Enzyme and bicarbonate concentration were in the range 150–300 μM and 40–128 mM, respectively. Inhibitor concentrations of 15, 30, 40 and 60 mM were investigated.

Samples for ^1H NMR measurements

Co(II)-substituted HCAI was obtained as described elsewhere [6]. The NMR measurements on the CoHCAI–imidazole adduct were performed in the pH range 6–9.5; the pH was varied by adding concentrated imidazole–sulfate buffer solutions at the appropriate pH, keeping the ionic strength constant with Na_2SO_4 as described above. Final enzyme concentrations were 1–2 mM.

NMR measurements

^{13}C NMR spectra were recorded using a Varian XL-200 spectrometer operating at 50.31 MHz for ^{13}C. D_2O was used for the internal field frequency lock. All runs were performed at 22.5 °C. Typical acquisition parameters were as follows: spectral width, 3 kHz; data points, 32 K; pulse delay, 4–8 s; acquisition time, 2 s; pulse width, 10 μs (40° pulse); collected number of scans, 700–5000, line broadening, 0.5 Hz. Proton noise decoupling was used throughout. The linewidths were measured directly from the chart tracings of the peaks. The linewidths of the $H^{13}CO_3^-$ resonance in solutions containing the fully inhibited enzyme by an excess of acetazolamide were 0.9–1.2 Hz. The linewidths obtained with the uninhibited enzyme over the range of bicarbonate concentrations investigated were 7.9–11.2 and 3.1–4.0 Hz at pH 6.8 and 8.8, respectively.

^1H NMR spectra were recorded on a Bruker MSL 200 instrument by using the modified DEFT pulse sequence [7]. Quadrature detection and a spectral width of 50 kHz were used; typical 90° pulse lengths were around 5.5 μs; recycle time was 90 ms. The T_1 values were obtained with the same pulse sequence by measuring the signal height M_τ as a function of τ. The data were best fitted to the equation [7]

$$M_\tau = M_\infty(1 - 2\exp(-\tau/T_1) + \exp(-2\tau/T_1))$$

with a non-linear two-parameter best fitting procedure. The estimated deviation is about 10%.

Kinetic measurements

At equilibrium and when the exchange is slow on the NMR time scale, the rate of $CO_2 \rightleftharpoons HCO_3^-$ interconversion, v_{exch}, is given by eqn. (1)

$$v_{exch} = \pi\,\Delta\nu_{HCO_3^-}\cdot[HCO_3^-] = \pi\,\Delta\nu_{CO_2}[CO_2] \qquad (1)$$

where $\Delta\nu$ is the line broadening due to the enzyme-enhanced exchange rate [8]. In our experiments such rates were always estimated from the HCO_3^- resonance. The classical Michaelis–Menten rate eqn. (2) was assumed

$$\frac{v_{exch}}{[E]} = \frac{k_{cat}^{exch}[S]}{K_{HCO_3^-}^{eff} + [S]} \qquad (2)$$

where [E] and [S] are the total enzyme and bicarbonate concentration, respectively; the kinetic parameters k_{cat}^{exch} and $K_{eff}^{HCO_3^-}$ (defined as the maximal exchange rate constant and an 'effective' substrate dissociation constant, respectively) [9, 10] were estimated from the Eadie–Hofstee plots in which the exchange rates were normalized to the enzyme concentration. These kinetic parameters determined from measurements at chemical equilibrium are related to the steady state kinetic parameters by eqn. (3) [9, 10].

$$k_{cat}^{exch}/K_{eff}^{HCO_3^-} = k_{cat}/K_m \qquad (3)$$

Results

Measurements of the CO_2/HCO_3^- exchange rates at equilibrium in the presence of imidazole from the linewidth of the bicarbonate resonance were performed at pH 6.8 and 8.8. The latter is the highest pH value at which the measurements were meaningful, since too small enzyme-induced increases of the linewidth were found at higher pH. Though this kind of measurements through ^{13}C NMR have proved to give reliable kinetic information [8], they

have seldom been employed in inhibition studies of CA, probably because of the rather elaborate preparation of the NMR samples. By using the simplified experimental technique described above, we have obtained the rate data reported in Fig. 1. At both pH values imidazole shows a competitive inhibition pattern. The parameters obtained from the least-squares regression analysis are as follows

pH 6.8: $K_{eff}^{HCO_3^-} = 19$ mM, $k_{cat}^{exch} = 6.7 \times 10^4$ s^{-1},

$K_i = (38 \pm 2)$ mM

pH 8.8: $K_{eff}^{HCO_3^-} = 250$ mM, $k_{cat}^{exch} = 1.9 \times 10^4$ s^{-1},

$K_i = (27 \pm 2)$ mM

The affinity constant values are in good agreement with that obtained from steady state measurements at pH 7 ($K_i = 50$ mM) [1] and with those obtained from the assumed competition of imidazole with iodide ($K_i = 20$–50 mM in the pH range 7–9) [2], and confirm the substantial pH independence of the affinity of imidazole for the native enzyme in the pH range 7–9. As far as the kinetic parameters of the CO_2/HCO_3^- interconversion are concerned,

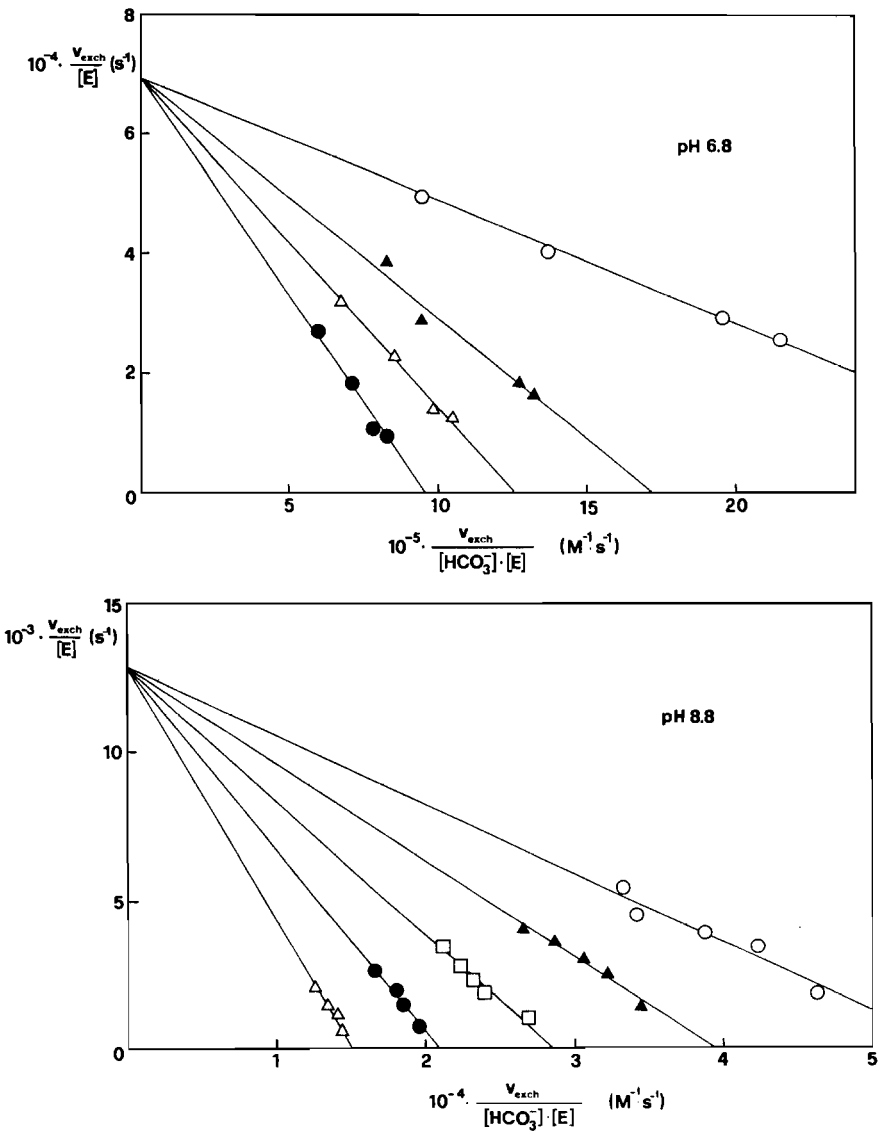


Fig. 1. HCAI-catalyzed CO_2/HCO_3^- exchange inhibited by imidazole. Inhibitor concentrations: pH 6.8: (○) 0 mM; (▲) 15 mM; (△) 60 mM; (●)120 mM. pH 8.8: (○) 0 mM; (▲) 15 mM; (□) 30 mM; (●) 40 mM; (△) 60 mM. Solvent was D_2O. pH meter readings are uncorrected for isotope effects. Ionic strength, 0.2 M (see text). Temperature, 22 °C. Lines were drawn to evidentiate the competitive behaviour of the inhibitor.

steady state measurements in the pH range 5.8–8.8 indicate that the exchange rate constant k_{cat} increases with increasing pH, while K_m is nearly constant [1]: the corresponding equilibrium values (k_{cat}^{exch} and $K_{eff}^{HCO_3^-}$, respectively) show an opposite pH behavior (Fig. 2), i.e. the exchange rate constant k_{cat}^{exch} is roughly constant while $K_{eff}^{HCO_3^-}$ increases with increasing pH; these data are in line with those previously obtained by Lindskog and coworkers [8, 9] and Koenig *et al.* [10, 11] from the same kind of equilibrium measurements. Furthermore, at both pH values, the ratio $k_{cat}^{exch}/K_{eff}^{HCO_3^-}$ is the same as previously observed [8] (at pH 8.8 the comparison has been made with an extrapolated value) and nearly identical to the corresponding value from steady state measurements [1], as it should be.

The hyperfine-shifted signals in the ^1H NMR spectrum of the high pH form of the Co-HCAI–imidazole adduct in the presence of sulfate are reported in Fig. 3(A) (sulfate was used as a counterion, like in the kinetic measurements). Three signals are observed, one of intensity 2. They are due to the three exchangeable histidine NH protons and to the 4H of His-119 (signal c), that is the only histidine bound to the cobalt ion through the N_1 nitrogen. In the high pH spectrum of the uninhibited enzyme (Fig. 3(B)) three signals are still observed, one of which is again of intensity 2. The absence of new signals due to the metal-bound imidazole over the 200 to −200 chemical shift range indicates that the chemical exchange of the inhibitor is fast on the NMR time scale (as it could be expected from its low affinity for the enzyme). Figure 3(G) and (E) show the ^1H NMR spectra of the Co-HCAI–imidazole adduct at pH 6 in the presence of sulfate and MES buffer, respectively. Also reported are the low pH spectra of uninhibited enzyme in unbuffered solution (Fig. 3(C)), in the presence of MES buffer (Fig. 3(D)) and in the presence of sulfate (Fig. 3(F)). These experiments were performed in order to check the possible interaction of sulfate with the adduct, since it is known that sulfate may bind the metal ion at low pH. Indeed, at pH 6.2 the CoHCAI–imidazole adduct in the presence of sulfate shows spectral features that differ from those of the enzyme in the absence of imidazole, whether unbuffered or in the presence of sulfate or MES. Also sulfate alone modifies the spectrum of the free enzyme. These data suggest that imidazole and sulfate are able to bind simultaneously to the low pH form of the enzyme. The spectrum of the sulfate–free imidazole adduct at pH 9.5 is nearly identical to that reported in Fig. 3(A) in the presence of sulfate. As expected, the enzyme–imidazole adduct at high pH can be formed independently of the presence of sulfate. Probably the adduct loses the ability of interacting with sulfate, analogously to the behavior of the pure enzyme.

The isotropically-shifted signals of the Co-HCAI–imidazole adduct in the presence of sulfate

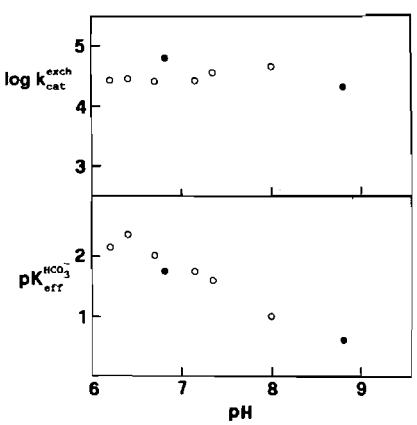


Fig. 2. pH dependence of the kinetic parameters for the CO_2/HCO_3^- exchange catalyzed by HCAI. (●) values from the present investigation; (○) values from ref. 8. pH meter readings are uncorrected for isotope effects.

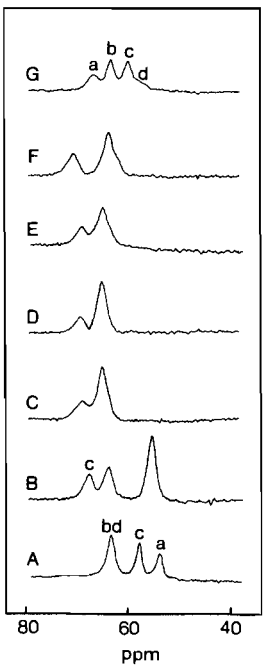


Fig. 3. Hyperfine-shifted signals in the 200 MHz ^1H NMR spectra of CoHCAI in H_2O. (A) CoHCAI–imidazole adduct in the presence of sulfate, pH 9.5; (B) unbuffered CoHCAI, pH 10; (C) unbuffered CoHCAI, pH 6.0; (D) CoHCAI in the presence of MES buffer at pH 6.2; (E) Co-HCAI–imidazole adduct in the presence of MES buffer, pH 6.2; (F) CoHCAI in the presence of sulfate, pH 6.2; (G) CoHCAI–imidazole–sulfate adduct, pH 6.2. Protein concentration, 2 mM; imidazole concentration, 500 mM; sulfate concentration, 230 mM.

are sensitive to pH. The pH dependence is reported in Fig. 4. The data are consistent with a pK_a of about 8 to which a further acid–base equilibrium at low pH is superimposed. The T_1^{-1} values of these signals at pH 6.2, 7.2 and 9.5 are reported in Table 1. It appears that there is no substantial change in T_1^{-1} values between pH 9.5 and 7.2, i.e. across the pK_a value of 8, whereas there is a sizable decrease in T_1^{-1} values between pH 7.2 and 6.2.

Discussion

Imidazole binding to carbonic anhydrase is still not well understood. It is known that two forms of the enzyme–imidazole adduct are present for the Co-substituted derivative [4, 12, 13]. They seem to be related by a deprotonation with a pK_a of about 8 [4, 12]. Accordingly, the affinity constant of imidazole is slightly pH dependent with the same pK_a [4]. For the native Zn enzyme the presence of two pH dependent species has not been demonstrated. Indirect measurements of binding constants do not show any major pH dependence [2]. Imidazole was shown to inhibit the activity of the native enzyme at pH 7 [1]. It was also shown that the inhibition was of a competitive type with respect to the CO_2 substrate. Our data show that the inhibitory efficiency of imidazole for the native enzyme is maintained at high pH. The behavior is still of a competitive type although our kind of measurements does not discriminate between competition with CO_2 or HCO_3^- or both.

In order to gain further insight on the imidazole binding mode, we have extended our previous investigations on the imidazole adduct of the Co-substituted derivative [12]. The well documented existence of two distinct adducts in a pH-dependent equilibrium for the latter derivative cannot be interpreted unambiguously [3, 4]. Indeed both Schemes 1 and 2 formally agree with the experimental findings. In Scheme 1 the ionization is attributed to the metal-bound water, while in Scheme 2 it is attributed to the exogenous ligand. The latter scheme has the advantage of allowing a pseudotetrahedral geometry for the high pH species, in agreement with the high intensity of the electronic spectra [3], and of framing imidazole binding in the general picture of neutral and anionic inhibitor binding to CA [3, 4]. The electronic spectra of the low-pH form of the HCAI–imidazole adduct in the presence of sulfate suggest a five-coordinate geometry [12]. In the light of the present results, the low pH form could be

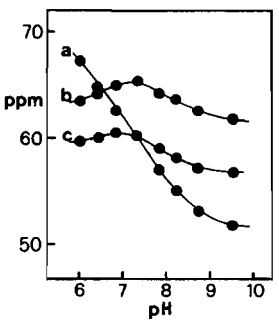
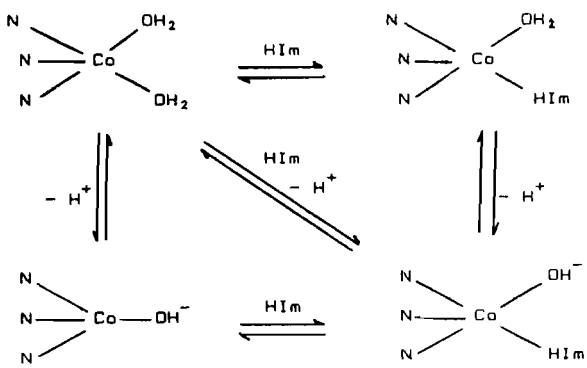


Fig. 4. pH dependence of the hyperfine-shifted signals in the spectrum of CoHCAI–imidazole adduct in the presence of sulfate. Protein concentration, 2 mM; imidazole concentration, 500 mM; sulfate concentration, 230 mM. See Fig. 3(A) and (G) for signal labelling.

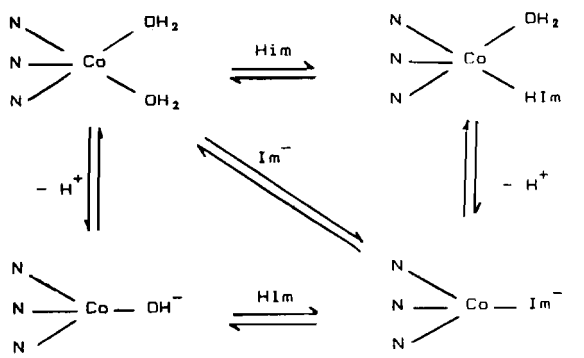
TABLE 1. Longitudinal relaxation rates for the isotropically-shifted signals of the CoHCAI–imidazole adduct in the presence of sulfate[a]

Signal	$T_1^{-1}\times10^{-2}$ (s^{-1})		
	pH 6.2	pH 7.2	pH 9.5
a	2.1	3.1	3.5
b	1.7	3.9	4.1
c	1.4	3.1	2.9

[a]See Fig. 3(A) and (G) for signal labelling; the estimated error is ±10%.



Scheme 1.



Scheme 2.

constituted by a ternary enzyme–imidazole–sulfate complex, whose formation occurs at pH values somewhat lower than the previously reported pK_a value of about 8. In an early X-ray investigation of carbonic anhydrase and some of its inhibitor derivatives it was proposed that imidazole could bind to the native metal ion in a loose fashion without displacing the coordinated water molecule [14, 15]. The resulting complex could be regarded as a distorted five-coordinated compound. Imidazole was believed to occupy an hydrophobic pocket in the inner region of the active site cavity. This behavior is, however, contrasted by several other CA ligands which give rise to pseudotetrahedral chromophores. The latter behavior, first proposed on spectroscopic grounds [3], is now confirmed by X-ray data for the important class of sulfonamide inhibitors [16].

Careful inspection of the active site of both CAI and CAII by computer graphics shows that imidazole could fit quite well the position occupied by the OH group in the pure enzyme, provided that the non-coordinated nitrogen is deprotonated and therefore hydrogen bonded to the OH proton of Thr-199. It is interesting to note that the nitrogen–nitrogen distance in the imidazole ring is very similar to the nitrogen–oxygen distance in the cyanate ion, which could also bind in this position [17] and indeed gives rise to a pseudotetrahedral chromophore in the Co^{2+} derivative [3]. The apparent discrepancy between the electronic spectra, that indicate a pseudotetrahedral geometry of the metal binding set in the high pH form of the CoHCAI–imidazole adduct [4, 12] and the early X-ray data [14, 15] might be reconciled by the observed effect of sulfate at low pH. The ^1H NMR shifts of the imidazole ligand itself, that, although small, are a sensitive reporter of the interaction of the ligand with the paramagnetic center, are significantly different from those of a blank solution containing Zn–HCAI (Table 2). In addition, the low pH ^1H NMR spectrum of uninhibited CoHCAI in the presence of sulfate (Fig. 3(F)) is markedly different from that of the free enzyme (Fig. 3(C)) and from that of the Co-HCAI–imidazole–SO_4^{2-} system at the same pH (Fig. 3(G)). Therefore it appears that *both* imidazole and sulfate are bound to the metal ion. Consistently, the proton relaxation rates of the protein ligands are smaller and the intensity of the electronic spectrum is smaller (as expected for an increase in the coordination number from four to five) as compared to the sample obtained in the absence of sulfate. The fact that the ^1H NMR spectrum of Co-HCAI–imidazole in the absence of sulfate (with MES buffer) at low pH (Fig. 3(E)) is very similar to that with only the buffer present (Fig. 3(D)) further suggests that the binding ability of imidazole at low pH would be smaller if it were not enhanced by sulfate. The position of the imidazole ligand in the active cavity in this ternary adduct could be very similar to that found by the X-ray investigation. It is interesting to note that the X-ray structure, although obtained at higher pH, was carried out on enzyme crystals soaked in very concentrated ammonium sulfate solutions [15]. It is a fact that five coordination can be indeed achieved in the active cavity of CA, and that the low pH form of the imidazole adduct is spectroscopically consistent with five coordination. The possibility of reaching five coordination, first proposed on spectroscopic grounds [3], is confirmed by a recent X-ray structure of the NCS^- adduct of HCAII [16]. Interestingly, the orientation of the NCS^- ligand is almost superimposable to the imidazole ring plane orientation proposed in the earlier structure. In both cases the water molecule is still coordinated to the metal, although its orientation is more toward the entrance of the cavity.

TABLE 2. Chemical shifts of the ^1H NMR signals of imidazole in the presence of sulfate at pH 6.2[a]

	2H	4H, 5H
Absence of enzyme	7.46	6.80
+Zn–HCAI (1 mM)	7.48	6.82
+Co–HCAI (1 mM)	7.68	7.06

[a]Imidazole concentration = 500 mM; sulfate concentration = 230 mM.

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