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PROTON MAGNETIC RESONANCE STUDIES OF HISTIDINES IN HUMAN, RHESUS MONKEY, AND BOVINE CARBONIC ANHYDRASES

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Summary

Histidine C-2 proton resonances in rhesus monkey carbonic anhydrase B (carbonate hydro-lyase, EC 4.2.1.1) and bovine carbonic anhydrase were investigated using 270-MHz proton magnetic resonance. The results suggest that there are extensive three-dimensional homologies between the human B and rhesus B enzymes and between the human C and bovine enzymes.

Resonances from solvent exchangeable protons have been observed in the 11—16 ppm range in the NMR spectra of human carbonic anhydrases B and C and bovine carbonic anhydrase. Up to five of these are sensitive to changes of pH and the presence of inhibitors. Three of these resonances are assigned to NH protons of the metal coordinated imidazole groups. These results are discussed in relation to various models for the catalytic mechanism of carbonic anhydrase.

Introduction

NMR is capable of giving both structural and dynamic information about a molecule in solution. The technique should therefore be uniquely well suited to test and extend models of enzyme mechanisms derived from X-ray crystallographic and enzyme kinetic data. An obvious example of this would be the determination of the nature, and the pK_a value, of an important catalytic group. Unfortunately the method has rarely led to a completely unambiguous result in this type of study. An example of a case where the NMR results have led to quite different interpretations is carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1), a zinc enzyme which catalyzes the interconversion of CO_2 and HCO_3^- [1].

Lindskog and Coleman [2] critically discussed several possible mechanisms for carbonic anhydrase and came to the conclusion that the most likely expla-

nation for the observed pH dependence of the kinetics was a zinc-coordinated water molecule which ionises around pH 7. This model received support from high resolution NMR studies by Campbell et al. [3,4], who concluded that a histidine residue was not the important catalytic group. However, in a similar study Pesando [5] proposed a mechanism involving a metal-coordinated imidazolate anion as the reactive group in CO_2 hydration. In addition, Gupta and Pesando [6] observed by NMR an imidazole ring NH proton which was sensitive to inhibitors and pH changes. They used this result to support the imidazolate model. We have carried out detailed studies of several exchangeable protons in the 12—16 ppm range. Our results are difficult to reconcile with the imidazolate model but are fully consistent with a zinc-hydroxide mechanism.

Materials and Methods

Enzymes and chemicals

Human carbonic anhydrases B and C were prepared by the method of Henderson and Henriksson [7] and bovine carbonic anhydrase was purchased from Sigma Ltd. The isoenzymes CI and CII (formerly called B and A [1]) were not separated, but these differ only by a single amino acid residue in a surface position [8]. Carbonic anhydrase B from the rhesus macaque was a gift from Dr. R.E. Tashian, Ann Arbor, Michigan, U.S.A. The enzymes were dialysed against 1 mM 1,10-phenanthroline at pH 7 for 48 h to remove paramagnetic metal ion impurities. All other chemicals were Analar grade and used without further purification.

Preparation of samples

The enzyme concentration used throughout was approximately 10% by weight (about 3.4 mM) unless otherwise stated. Enzyme concentrations were determined spectrophotometrically [9]. The ionic strength was maintained at 0.1 M using Na_2SO_4. The pH was measured using an Activion combination electrode incorporating a K_2SO_4 salt bridge unless otherwise stated. For the pH titrations two stock solutions were prepared, one at high pH and one at low pH. Protein solutions of intermediate pH were obtained by mixing the appropriate amounts of the stock solutions, thus maintaining the same enzyme concentration throughout. For the histidine C-2 proton measurements the solvent was 99.8% ^2H_2O, and for the low field NH measurements the solvent was 90% ^1H_2O and 10% ^2H_2O. The sample volume was 0.4 ml in both cases. All pH values quoted correspond to the uncorrected meter readings.

NMR measurements

The measurements on the C-2 proton resonances were carried out at 270 MHz as described previously [3], and the chemical shifts are quoted as ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate. The measurements of the exchangeable NH resonances were made by applying a long, selective pulse to suppress the strong ^1H_2O peak just before the normal observation pulse in the Fourier transform mode [10]. The 10% ^2H_2O in the samples were used for the field-frequency lock. A typical spectrum is shown in Fig. 1.

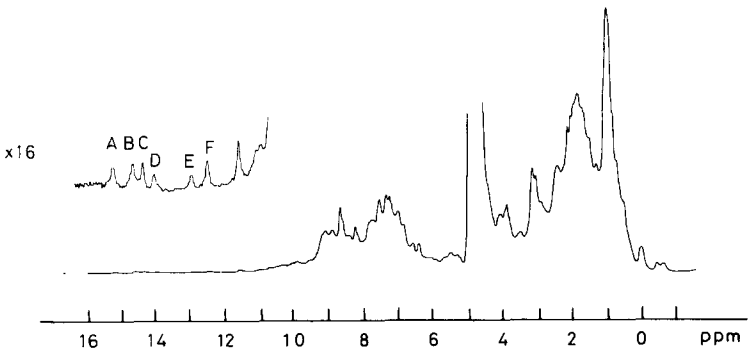


Fig. 1. The 270-MHz Fourier transform ^1H NMR spectrum of 3.5-mM human carbonic annydrase C in 90% ^1H_2O, 10% ^2H_2O, pH 6.6, 25°C. The strong ^1H_2O peak has been suppressed using a selective pulse applied at the ^1H_2O frequency [10].

Results and Discussion

Histidine C-2 proton resonances

Campbell et al. [3,4] observed three "nontitrating" resonances in the human B and C enzymes which were sensitive to the presence of inhibitors and changes of pH. These resonances were assigned to the C-2 protons of the metal-coordinated histidine residues. Since the nature of the ligands is likely to be an invariant feature of the active site, these resonances should also be present in the NMR spectra of carbonic anhydrases from other sources, and they might be sensitive indicators of subtle species differences in the structure of the metal centre.

The pH dependencies of resonances in the histidine C-2 proton region of the NMR spectra of rhesus carbonic anhydrase B and bovine carbonic anhydrase are shown in Fig. 2 and Fig. 3, respectively. Three "nontitrating" resonances (labelled 1—3) are, indeed, observed in both cases. The rhesus B enzyme resonances behave rather like the corresponding resonances in the human B enzyme [3] whereas the bovine enzyme resonances are similar to those of the human C enzyme [4]. Resonance 1, which was assigned to the ligand His 119 *, is shifted about 0.3 ppm to higher field in the bovine enzyme compared to the human C enzyme. In the rhesus B enzyme resonance 1 is about 0.2 ppm to lower field compared to the human B enzyme. These observations are in accordance with previous conclusions from spectroscopic properties of the Co(II) derivatives that there are small species differences in the metal ion coordination [1].

The titratable resonances 5—8 of the rhesus B enzyme are very similar to resonances 5—8 in the spectrum of the human B enzyme (see Fig. 3 in ref. 3). The major difference in the NMR patterns of these two enzymes is the absence in rhesus B of resonance 4 in human B [3]. This resonance was tentatively assigned to His 243, which is located on the surface of the human B enzyme

* Residue numbers indicate the sequence position using the numbering of human carbonic anhydrase B [11]. To convert to the numbering of the human C enzyme sequence subtract 1 from positions 2—125 and 2 from positions 127—261 [12].

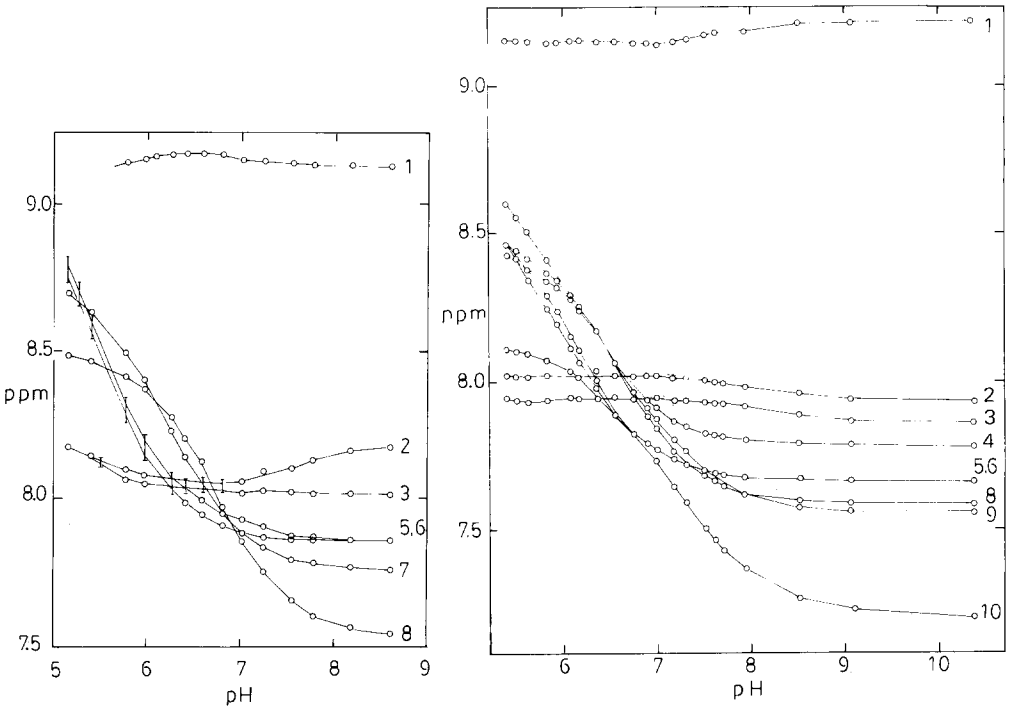


Fig. 2. pH titration data for imidazole C-2 ^1H resonances in carbonic anhydrase B from the rhesus macaque in ^2H_2O, 25°C. The numbering has been chosen to illustrate similarities with the human B enzyme [3].

Fig. 3. pH titration data for imidazole C-2 ^1H resonances in bovine carbonic anhydrase in ^2H_2O, 25°C, in the presence of 25 mM I^-. The numbering has been chosen to illustrate similarities with the human C enzyme [4].

molecule. The rhesus B enzyme has an Arg in this sequence position [13]. Thus, the absence of resonance 4 in Fig. 2 adds support to the previous assignment. The only additional difference in sequence positions of histidines in these two enzymes is a replacement of Gln 165 in the human B enzyme with His in the rhesus B enzyme [13]. The resonance corresponding to His 165 was not observed in the spectrum of the rhesus enzyme, but it is possible that this resonance titrates very closely together with resonances 5 and 6. In addition, resonance 9 in the human B enzyme, which corresponds to a titratable active site histidine, was not detected in the rhesus B enzyme. However, this resonance is difficult to observe in the human enzyme because of its low pK_a, its linewidth and its position within the main aromatic envelope. Unfortunately the supply of rhesus B enzyme was not sufficient for extended investigations to search for these resonances. It should also be noted that a KCl bridge was used in the rhesus B study; thus the apparent pK_a of the activity-linked group will be too high [3].

The titration of the bovine enzyme was made in the presence of 25 mM I^-, because at this condition the maximum number of resolved resonances were observed in the human C enzyme. The results in Fig. 3 should be compared with those for the human C enzyme in the presence of I^- (Fig. 3 in ref. 4).

These two enzymes have very similar patterns, suggesting that the tertiary structures are quite homologous despite a 21% nonidentity in the primary structures [14]. The only difference with respect to sequence positions of histidines is a replacement of His 36 in the human C enzyme with Lys in the bovine enzyme. Resonance 7 in the human C enzyme was tentatively assigned to His 36 [4], and this resonance is absent in the spectrum of the bovine enzyme confirming the earlier assignment.

The only remaining significant difference is that resonance 4 has a higher pK_a value in the bovine enzyme (about 6.1) than in the human C enzyme (5.48, [4]) and a slightly different shift position. This resonance, together with resonances 5, 6, 8, and 9, was assigned to a group of five surface histidines located in the N-terminal portion of the sequence. The environments of these surface histidines should be very similar in the two enzymes, because the N-terminal sequences of the bovine and human C enzymes are almost identical. Further, model studies [15] show that these histidines do not interact with groups derived from other segments of the peptide chain. However, the single difference in the first 30 sequence positions is a substitution of Asn in the bovine enzyme for Lys 24 in the human C enzyme. The model studies suggest that Lys 24 is free to interact with His 17, and this might explain the lower pK_a observed for resonance 4 in the human C enzyme. Therefore, we tentatively assign resonance 4 to His 17.

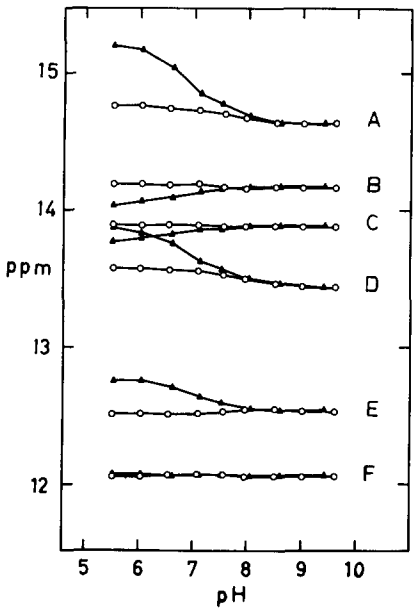
Resonance 10 corresponds to the only titratable active site histidine in the human C enzyme [4]. It is noteworthy that this resonance has a very similar chemical shift and pK_a value in the bovine enzyme.

NH resonances

Figs. 4, 5, and 6 show the pH-dependence of the exchangeable resonances observed below 12 ppm in the human C and B carbonic anhydrases and in the bovine enzyme, respectively. It is probable that these arise from the NH protons in hydrogen bonded imidazole groups (see, for example, ref. 16). In addition to resonances A—F there are several resolved, exchangeable resonances to higher field. There are some resonances in the vicinity of F which appear at some pH values (see ref. 6), but these have no obvious pattern of behaviour.

In all cases resonances A and E move to high field on the addition of I^- at low pH, while resonances B and C move to low field on the addition of I^-. Resonance F is insensitive to pH or inhibitor. Resonances A to E show a pH inflection at approximately pH 6.9 in the human C enzyme, 7.0 in the bovine enzyme, and 7.4 in the human B enzyme. In the human B enzyme resonances A and E are most sensitive to the presence of imidazole.

The method used to observe these resonances [10] can result in transfer of saturation from the ^1H_2O resonance, if the rate of chemical exchange between the observed resonance and the solvent is in the 100-ms time range. This effect reduces the observed intensity of certain peaks, and resonances D and E sometimes display this effect (see Fig. 1). If the lifetime of the hydrogen bonded proton is 1 s or longer, no reduction of peak height is expected; resonances A, B, C, and F appear to come into this category. For protons with lifetimes less than 10 ms severe broadening can result from exchange with the solvent, and for still faster exchange no resonance will be observed. Resonance D is not



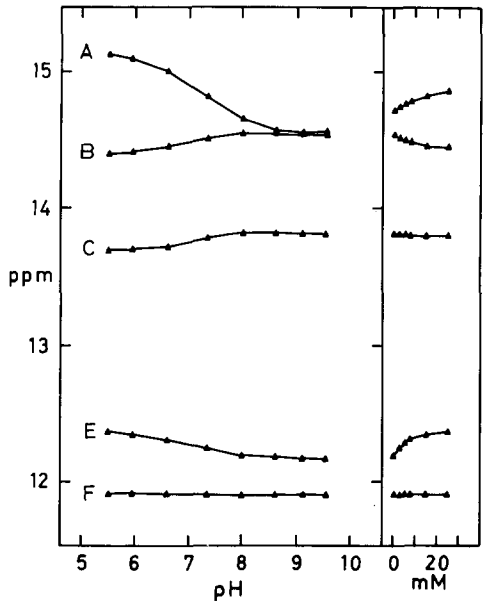


Fig. 4. The pH dependence of the chemical shifts of the exchangeable resonances labelled A—F in Fig. 1. (▲), 4 mM human C enzyme (○), 4 mM human C enzyme plus 20 mM I^-.

Fig. 5. (A) As in Fig. 4. (▲), 4 mM human B enzyme. (B) Effect of adding imidazole at pH 7.9.

observed in the human B enzyme or in the bovine enzyme at high pH.

The evidence available for assigning the resonances A to F may be summarised as follows. 5 of these resonances are sensitive to pH and I^- in the

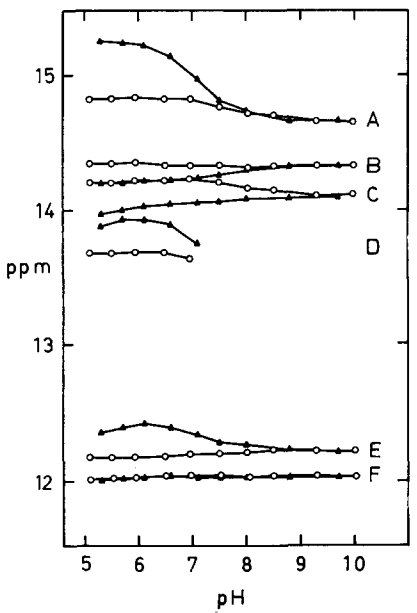


Fig. 6. As in Fig. 4. (▲), 4 mM bovine enzyme; (○), 4 mM bovine enzyme plus 20 mM I^-.

human C enzyme and the bovine enzyme. In the human B enzyme only 4 such sensitive resonances are observed. The observed inflections in the pH dependencies of resonances A—E correlate with the observed pH vs. activity profiles within experimental error. It is thus likely that these resonances reflect the ionisation of the activity-linked group just like the three "nontitrating" C-2 proton resonances [4]. Therefore, it seems reasonable to assign three of the sensitive resonances to the ring NH protons of the metal-coordinated imidazole groups. Resonance A is most sensitive to pH and I^-. Resonances D and E appear to exchange more rapidly with solvent than the others. In addition, Gupta and Pesando [6] have shown that resonances B and C are comparatively insensitive to major changes at the metal site, for example in the apo or cobalt enzymes. These data allow the tentative assignments of resonances A, D, and E to the ligand NH protons.

Resonance D, which is not observed in the human B enzyme and broadens in the bovine enzyme, could be from His 94 which is the most exposed of the three ligands [15,17] and thus most likely to exchange with solvent. Imidazole, which inhibits the human B enzyme [18] binds near the metal ion [19] and causes a pronounced upfield shift of the C-2 proton assigned to His 94 [3]. From the known structure of the imidazole complex [19] it can be predicted that the NH proton of His 94 would also undergo an upfield shift upon imidazole binding. Such an effect is not observed in the spectrum. Although this evidence is negative, it is consistent with the assignment of resonance D to His 94. His 119 is the least exposed of the ligands. Resonance A could be from His 119, the C-2 proton of which is observed to low field of the other two ligand C-2 proton resonances [3,4] (compare also Figs. 2 and 3). Resonance E could then be from His 96.

Resonances B and C, although probably not from ligands, must be from protons in the vicinity of the active site, since they are sensitive to the activity-linked ionisation and to the binding of inhibitors. One possible candidate is His 107 which is close to His 119 and involved in a hydrogen bonded network to Glu 117 and Tyr 194 (see Fig. 7). There is one other histidine which is not exposed to the solvent, His 122, but this is probably too far away from the zinc ion to be influenced by the catalytic group; this could give rise to an unresponsive resonance such as F. It thus seems reasonable to assign either B or C to His 107. The assignment of the other resonance is then a problem, since if all the resonances in the 12—16 ppm range come from histidines, and if His 107 is neutral as would be expected at pH 10, there are apparently 6 resonances from 5 histidine NH protons (His 94, 96, 107, 119, 122). Let us reconsider the assumption that all the resonances are from histidines. Tryptophan indole NH protons have not been so well studied, but these have been observed as low as 11 ppm in lysozyme [20] and 11.9 ppm in *Escherichia coli* thioredoxin [21]. Hydrogen bonded OH resonances, for example in salicylaldehyde, resonate around 11 ppm, and carboxylic acid dimers resonate around 12 ppm. The most likely possibilities, therefore, appear to be that the extra resonance is from a strongly hydrogen bonded -OH proton, from a tryptophan indole -NH, or from a second proton on His 107. After studying the crystal structures of Kannan et al. [17] and Vaara [15] we consider the most likely candidate to be the OH proton of Tyr 194, because this proton appears to be strongly hydrogen

bonded to be imidazole group of His 107. Another possibility is the indole NH proton of Trp 209, which is the hydrogen bonded to Ser 29. These tentative assignments are summarised in Fig. 7.

Implications for the catalytic mechanism

There is strong evidence that the titratable catalytic group in carbonic anhydrase is closely linked to the metal ion [1,2]. As discussed in detail above we observe 8 proton resonances which appear to reflect the behaviour of this group. 6 of these are assigned to the C-2 and NH protons of the three metal-coordinated imidazole groups. Despite many efforts the chemical nature of the catalytic group has not been conclusively identified. Four distinct alternatives have been proposed: (1) a metal-coordinated water molecule ionising to OH^- [1,2]; (2) a titratable imidazole group associated with the metal ion via one or more water molecules [22]; (3) a neutral imidazole group ionising to a metal-coordinated imidazolate anion [5,6,23]; (4) a partially buried carboxyl group connected to a metal-coordinated water molecule via a hydrogen bond system [19].

In two previous papers [3,4] we have shown that the titratable, active-site histidines of human carbonic anhydrases B and C behave independently of the catalytic group. Alternative 2 can, therefore, be excluded. The function of these histidines is unknown. It has been proposed that His 64 might facilitate the transfer of protons between the metal centre and the solvent [24]. Recent work by Whitney and Brandt [25] and by Strader and Khalifah [26] shows that the titration of His 200 in the human B enzyme has certain effects on inhibitor binding and catalytic activity. This interdependence of His 200 and the catalytic centre appears to be reflected in some of the proton resonances as well. The ligand C-2 proton resonances 1—3 in the human and rhesus B enzymes are sensitive to pH not only around the pK_a of the catalytic group but

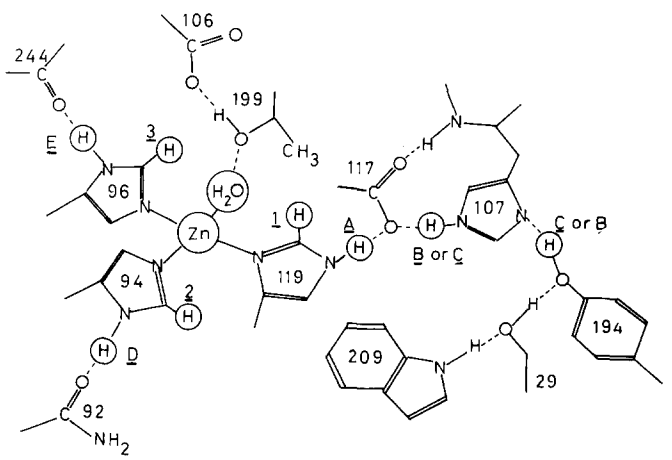


Fig. 7. A schematic representation of part of the crystal structure of carbonic anhydrase, showing the three imidazole zinc ligands and the hydrogen bonding network around the active site. These features are common to the human carbonic anhydrases B and C [17]. The assignments of resonances 1—3 and A—E are indicated.

also between pH 5 and 6 (cf. Fig. 2) which is in the range of the titration of His 200 [3,25—27].

The imidazolate anion model (alternative 3) was based essentially on one piece of evidence, namely a downfield shift of resonance 2 at increasing pH in the human B enzyme [5]. However, this feature is not common to all carbonic anhydrases; we observe it in the human and rhesus B enzymes, but this behaviour is not observed in the human C and bovine enzymes. Our assignments of the pH and inhibitor sensitive NH resonances are not compatible with the imidazolate model. As discussed by Gupta and Pesando [6], the NH proton of a catalytically functional ligand must be in rapid exchange with the solvent and would not be observable in the NMR experiment. Our interpretation seems supported by the crystal structure studies suggesting that all the ligand NH groups are, indeed, involved in hydrogen bonds [17]; His 94 to Gln 92; His 96 to the peptide carbonyl of Asn 244; His 119 to Glu 117 (Fig. 7). In view of these considerations the imidazolate model seems unlikely, although the NMR evidence might not be unambiguous enough to exclude it. Thus, alternatively it is possible to assign resonances A—E to protons from only two of the ligands, His 107, Tyr 194, and Trp 209.

Recently Kannan et al [19] proposed that the carboxyl group of Glu 106 might function as the titratable, catalytic group (alternative 4). Glu 106 forms a strong hydrogen bond with Thr 199, which is hydrogen bonded to the metal-bound water molecule (or OH^-). Clearly, this structural feature must be of importance for the properties of the catalytic centre. However, this model requires that Glu 106 has an exceptionally high pK_a value despite the close contact with the positively charged metal ion. In addition, the NMR results may be interpreted as evidence favouring the zinc-hydroxide model over the Glu 106 model. The addition of OH^- or an anionic inhibitor at low pH cause very similar upfield shifts of the ligand NH proton resonances A, D, and E. Gupta and Pesando [6] concluded that such shifts could arise from a decrease of the net charge on the zinc ion. In the zinc-hydroxide mechanism OH^- and anionic inhibitors compete for the same coordination site on the metal ion, while pH and anions would have different effect on the localization of charges in the Glu 106 model. Thus, our results are in complete accordance with a zinc-hydroxide model for the catalytic mechanism of carbonic anhydrase, whereas alternative models appear less attractive.

Acknowledgements

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