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The Acid–Base Equilibria of Carbonic Anhydrase

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The electronic spectra of cobalt substituted bovine and human carbonic anhydrase B have been recorded both in ion free solutions and in presence of 'non interacting' buffers, at different pH values.

The water proton longitudinal relaxation times in solution of the cobalt substituted human enzyme have also been measured as a function of pH in unbuffered solutions at 80 MHz. The above data, together with previous experimental evidences, are discussed in terms of the possible ionizations occurring in the active site of the enzyme.

Introduction

The cobalt substituted carbonic anhydrase is an active enzyme [1] which can be investigated with confidence as a model for the native zinc containing enzyme. The d–d spectra of the cobalt(II) derivative, which is high spin, are well shaped and markedly pH dependent [2–4]; the catalytic activity of both the native and cobalt substituted enzymes also depends on pH [5]. The pH dependences of both the spectra and the catalytic activity have always been attributed to a single acid–base equilibrium [6–9].

Anionic ligands act as inhibitors of the enzyme by binding at the metal ion [6]. The affinity constants of inhibitors for the enzyme also depend on pH, with profiles which are commonly assumed to be of sygmoidal type with a pK_a equal to that of the acid–base equilibrium of the enzyme [8]. However, it had been noted that the pH dependence of the iodide affinity constant for the human B enzyme does not fit in the above frame [10], and that for some inhibitors of the cobalt bovine enzyme such affinity constants increase, by lowering the pH to values below 6, much more than expected on the basis of a single dissociating group [11].

Another experimental evidence of a more complex acid–base equilibrium displayed by the enzyme, which unfortunately has been generally overlooked, is given by an early work of Lindskog and Nyman, who measured the pH dependence of the stability constant of the zinc–apoenzyme complex [3]. The logK values increase almost linearly from pH 5 to pH 10, indicating that at least one group deprotonates at high pH, which is near enough to the zinc atom to affect its coordinating capability.

More recently, a ^{13}C NMR study on a chemically modified carbonic anhydrase has also pointed out the existence of more than one ionization in the active site cavity [12]; finally, a careful examination of hydrogen isotope effect on the catalytic activity of the human C enzyme has led to the assumption that two independently ionizing groups are involved in the catalytic reaction [13].

^1H NMR relaxation studies of water solutions containing paramagnetic centers have proved useful to detect exchangeable protons interacting with the paramagnetic center [14, 15]. In the case of the bovine enzyme such studies have shown that exchangeable protons actually interact with the cobalt ion and that the ^1H T_1^{-1} enhancements with respect to solutions of the diamagnetic enzyme are pH independent [16], provided they are measured in the absence of anions which interact with the metal ion. Therefore these exchangeable protons are not involved in acid–base equilibria between pH 5.8 and 9, which is the pH range investigated.

With this in mind we have accurately registered again the electronic spectra of both bovine B and human B cobalt enzymes (CoBCAB and CoHCAB hereafter) as a function of pH in unbuffered solutions and in presence of non interacting buffering systems; furthermore the ^1H relaxation rates of solutions of CoHCAB have been remeasured in absolutely ion free solutions in order to check the difference with the behavior of the bovine enzyme.

Experimental

Bovine carbonic anhydrase and human carbonic anhydrase B were obtained from Sigma. The bovine isoenzyme B was isolated through chromatography on DEAE cellulose [17]. Both enzymes were demetallized against two changes of pyridine-2,6-dicarboxylic acid 0.1 *M* in 0.1 *M* phosphate buffer,

pH 7.0 [18], and reacted with cobalt sulfate 10^{-3} *M*. Cobalt enzymes were extensively dialyzed against freshly bidistilled water, until the isoionic pH (*ca.* 5.6) was reached. Care was taken to exclude CO_2 from contact with the enzyme solutions.

Unbuffered samples for electronic and NMR spectra were prepared by adding increasing amounts of NaOH to the above solutions. The pH of the samples was always measured after recording the spectra *in situ* with a microelectrode. The buffered samples were obtained by mixing equal amounts of unbuffered enzyme solutions with solutions of 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES hereafter), to which different calculated amounts of NaOH had been added. The pH of each sample was again measured only after having recorded the electronic spectra and was found to be negligibly different from the calculated value. In such a way the samples went in touch with the electrode after the measurements, avoiding the risk of chloride leakage which could alter the results [19]. The human enzyme solutions were also degassed immediately before the measurements, in order to minimize the possibility of CO_2 uptake [20].

Electronic and NMR spectra were registered with the apparatus and techniques described elsewhere [16].

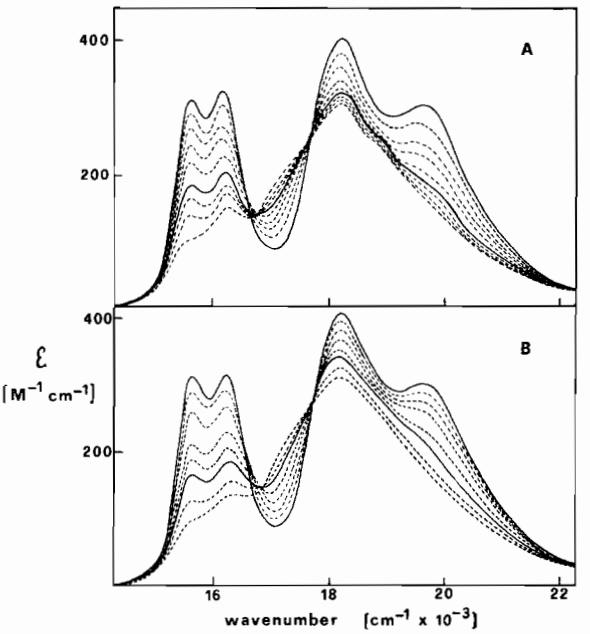


Fig. 1. Electronic spectra of CoBCAB as a function of pH. A: unbuffered solutions at pH 5.8, 6.0, 6.7, 7.3, 7.7, 7.9, 8.2, 8.8 in order of increasing ϵ_{640}; B: 0.1 *M* HEPES solutions at pH 5.9, 6.2, 6.5, 7.0, 7.3, 7.8, 8.4, 9.1 in order of increasing ϵ_{640}. The full lines represent the spectra obtained at the middle and at the end of the titration.

Results

Some electronic spectra of CoBCAB and CoHCAB are reported in Figs. 1 and 2, both in unbuffered solutions and in solutions buffered with HEPES. Apparently the spectra were not affected by HEPES whereas they are affected by other buffering species (phosphate, Tris sulfate, *etc.*) whose anions are less bulky and bind the metal ion [21, 22]. The strict similarity of the pH behavior of the spectra in buffered and unbuffered solutions also shows that ionic strength is not a major factor [23] affecting the acid–base equilibria.

The plot of the molar absorbance at 640 nm against pH does not follow a simple pattern as expected for a single dissociating group. In the case of the bovine enzyme (Fig. 3) the observed profile can be qualitatively reproduced with two dissociating groups with pK_a values <6 and >7, respectively. Of course even more acid–base equilibria with various extent of perturbations of the electronic spectra would improve the fitting of the experimental data. In the case of the human enzyme (Fig. 4) the profile shows a less marked inflection, but apparently also in this case several equilibria with different pK_a values, each of them capable to slightly affect the electronic spectra, qualitatively reproduce the observed pattern. It should be noted that for both isoenzymes the major change in the general shape of

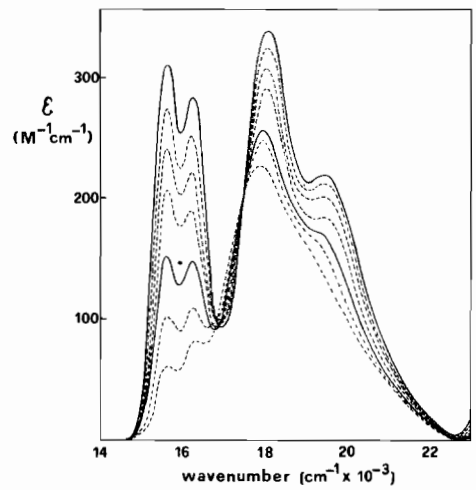


Fig. 2. Electronic spectra of CoHCAB as a function of pH in 10^{-2} *M* HEPES solutions at pH 6.1, 6.6, 7.1, 7.8, 8.3, 8.6, 9.5 in order of increasing ϵ_{640}. The full lines represent the spectra obtained at the middle and at the end of the titration.

the spectrum (*i.e.* the appearance of the low energy bands) occurs roughly within the first half of the titration, while the effect of a further increase of pH leads only to a general increase of the intensity. In the presence of most inhibitors, *e.g.* chloride, the ϵ_{640} profile *versus* pH is much closer to a single sygmoid

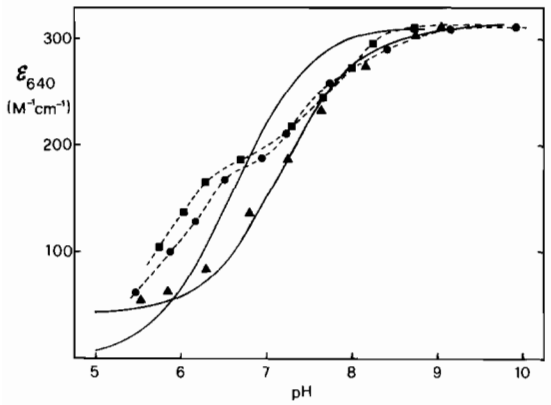


Fig. 3. Intensity of the 640 nm d–d transition of CoBCAB as a function of pH in unbuffered (■) and 0.1 *M* HEPES buffered (●) solutions. The solid line on the left is calculated for a single pK_a = 6.6. The triangles are the values obtained in 0.1 *M* NaCl, which are fitted to a single sygmoid with pK_a = 7.2.

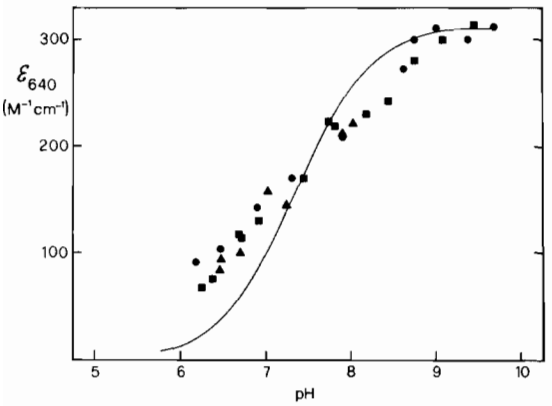


Fig. 4. Intensity of the 640 nm d–d transition of CoHCAB as a function of pH in 5 × 10^{-2} *M* (●), 1 × 10^{-2} *M* (■), and 5 × 10^{-3} *M* (▲) HEPES solutions. The solid line is calculated for a single pK_a = 7.35.

(Fig. 3). In fact the inhibited species do not generally absorb at 640 nm, and detachment of the inhibitor has to take place before any absorption at this wavelength shows up with increasing pH.

The ^1H T_1 values of water solutions containing CoHCAB show that at every pH there is sizeable T_1^{-1} enhancement with respect to the values measured in the presence of the diamagnetic zinc enzyme (Fig. 5). However, the enhancement at low pH is significantly smaller than at high pH, although such a variation is smaller than that previously reported [24].

Discussion

The present data definitely show that the pH dependence of the electronic spectra of the cobalt

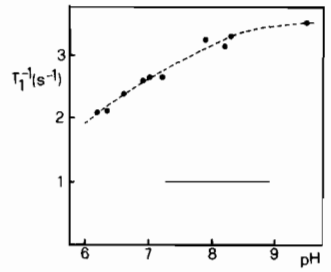


Fig. 5. Water proton T_1^{-1} values of a solution of CoBCAB 6.5 × 10^{-3} *M* at various pH values (●). The solid line represents the T_1^{-1} value obtained in the presence of the native enzyme in the same concentration.

substituted enzymes is not as simple as expected for a single dissociating group. Previous measurements were usually performed in buffered solutions, or in the presence of salts to keep the ionic strength constant [2–4]. All of these factors could be responsible for the 'simplification' of the titration curve.

The problem remains of whether the catalytic activity parallels the spectral dependence on pH. Unfortunately such activity measurements have to be performed in the presence of buffers which are not inert, especially if they are not as bulky as HEPES. Finally, an entire sequence of data with the same systematic error cannot be obtained for the range of pH under investigation since the hydration of CO_2 can be easily investigated only at pH >7 and the dehydration of HCO_3^- is conveniently investigated below pH 7. Therefore, minor deviations from a simple sygmoidal behavior are hard to recognize in such type of measurements.

The ^1H relaxivity data of solutions containing the paramagnetic metalloenzyme show that the suggested $H_2O \leftrightarrows OH^-$ equilibrium [8] is not consistent with the constancy (or increase at high pH values in the case of the human enzyme) of the T_1^{-1} enhancements, unless it is assumed that a decrease in the metal hydrogen distance as well as a change in the electronic relaxation times accidentally account for the observed data. Against the above hypothesis are the measurements of the other metallosubstituted enzymes [25–28], all of which show almost constant relaxation versus pH and make unlikely the repeat of such accidental cancellations. Therefore it seems probable that a water molecule is bound at every pH investigated; the difference in the ^1H T_1^{-1} enhancement between the low and high pH forms of CoHCAB can be attributed to a sensible variation of the electronic relaxation times for the two enzymatic species.

If H_2O is not one of the groups responsible for the variation of the electronic spectra, other possibilities should be taken into consideration. Up to now substantially three other mechanisms for the pH depen-

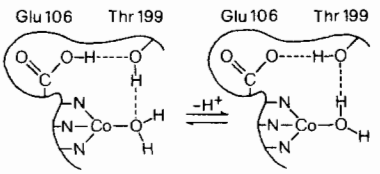
dence of the enzyme properties have been suggested: i) the ionization of a neutral histidine bound to the metal [29, 30], or alternatively the dissociation of a free histidine hanging into the cavity [31] ii) the dissociation of the Glu 106 residue [32] iii) addition of a fifth OH group at high pH [33].

The present results show that at least two dissociations take place within two pH units near the metal ion enough to perturb its electronic spectrum; therefore the main message of the present research is that there is no reason to ascribe all the pH dependent properties of the enzyme to only one dissociating group: one group could be responsible for some properties and some other for other properties, in such a way that a more complex explanation should be looked for.

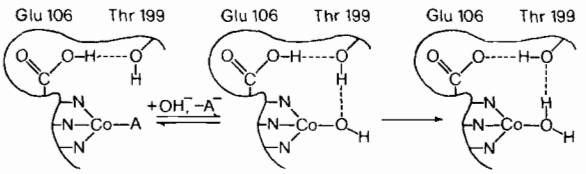
The pH dependence of the affinity constants of inhibitors, as well as the profile of the pH dependence of the electronic spectra in the presence of anions of the type of chloride should shed more light on this problem. In general the apparent affinity of anions decreases with pH. The same behavior is followed also by copper [25, 34] and nickel [26] carbonic anhydrase derivatives which, however, show only little catalytic activity. Since for the latter ions there are more clear evidences that there is no change in coordination number with pH [25, 26], the addition of OH^- is not presumably governing the pH dependence of K_{app}. The main attracting property of a metal bound hydroxide, either generated by the dissociation of the water molecule or added as a fifth ligand, was its capability to explain in a straightforward way the release of anions from the inhibited enzyme in terms of ligand competition: the stronger the anion binding, the higher the OH^- concentration needed to displace it from coordination. In the case of the cyanide ion the displacement may take place at pH >10; in other words, the pK_a of the inhibited form is raised of more than three units. Every other proposed ionizing group has to exhibit the same flexibility of the pK_a in order to be a reasonable candidate.

The first possibility to be considered is the ionization of one of the metal bound histidines: as in the $H_2O \rightleftharpoons OH^-$ case the decrease of the pK_a of the pyrrole type proton from about 14 to about 7 can be ascribed to the coordination to the bipositive metal [30] considered as a Lewis acid. The binding of anions would decrease the acidity of the complex according to their basic strength ($CN^- > HS^- > N_3^- > X^-$), shifting the pK_a of the pyrrole proton towards higher values.

Another attracting possibility is the dissociation of the Glu 106 residue which, as proposed by Kannan [32], would take place according to the following scheme; it could be consistent with the binding of weak acids like HCN, H_2S or sulfonamides, since the binding of the anion would result in donating a proton to the Glu residue through the hydrogen



bonding net, and in burying it within the protein. This scheme would account for a cyanide ion bound to the 'acidic' form of the enzyme even at pH as high as 10. Even in the case of anions of strong acids like halides the burying of a Glu group would be consistent with the shift of the pK_a towards higher pH. However, once the adduct of the ion with the acidic form of the enzyme is formed, a further mechanism is needed in order to allow the protonated Glu residue to ionize when the pH is increased, *i.e.* to be again connected with the solvent. This could be performed by a second dissociating group which would titrate independently of the Glu residue in the non inhibited enzyme and could trigger it when the inhibitor is present. Alternatively, the idea of competition with OH^- could again be considered: in this frame the incoming hydroxide ion would replace the anion, coordinate the metal, become hydrogen bonded to Thr 199, and abstract the proton from the acidic Glu residue regenerating a metal bound water molecule.



The reaction would be

$$R\text{-}COOH + OH^- \rightarrow R\text{-}COO^- + H_2O$$

It is worthy of note that the same mechanism could work for the catalytic cycle [32]: once the HCO_3^- product is formed, it binds the metal and it is subsequently displaced by an OH^- group, which needs an internal proton transfer from Glu 106 to regenerate the enzyme form which is active for the next cycle. The necessity of an internal proton transfer has been recently pointed out [13]. The supply of OH^- can be in turn accelerated by the presence of buffers, as has been recently proposed [35].

Either the Glu or the histidinate mechanisms are capable to overcome to a large degree the difficulties arising in the interpretation of the pH dependent properties of carbonic anhydrase, except for the electronic spectra of the cobalt derivative; if it is kept in mind that both dissociations are likely to occur without influencing too much each other, the variations of the electronic spectra of the cobalt enzyme with pH could be related to both dissociations. When

the inhibitor is bound both of them would be blocked, the GluH residue being disconnected from the solvent and the imidazole becoming a weaker acid, as discussed before; as a matter of fact the electronic spectra of all the enzyme–inhibitor adducts are pH independent [16]. Since the inhibitors bind when these two groups are protonated, their affinity constants would be no longer a simple function of pH, as has been previously noted [11]. Of course this is just a possibility which, however, does not try to account for the relation between catalytic activity and pH.

Up to now the discussion has been restricted to two possible dissociating groups which are present in both the human and bovine enzymes; there is only another group which has not been considered yet, namely Hys 63, which is in the active cavity of all the known isoenzymes and is also connected with the solvent structure. While this group cannot be responsible for the binding of inhibitors, since ^{1}H NMR studies on histidine protons have shown it to titrate both in the presence and absence of anions, although with slightly different pK_a [36], it is likely to further increase the affinity constant of anions at low pH [11] by increasing the positive charge within the cavity. His 63, therefore, may also have a role in the buffer mediated proton transfer step [35] required in the catalytic mechanism.

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