EVIDENCE OF EXCHANGEABLE PROTONS IN THE ACIDIC FORM OF MANGANESE(II) BOVINE CARBONIC ANHYDRASE B

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1. Introduction

Bivalent metal ions in the active site of carbonic anhydrase have been shown by X-ray analysis at 2.2 Å resolution to be bound to three histidyl nitrogens [1]. The coordination polyhedron around the metal atom is a distorted low symmetry one and for the native, zinc-containing enzyme, as well as for the cobalt analogue, a pseudotetrahedral structure has been proposed [2–4].

The fourth donor atom was suggested to be a water molecule [1,2]. However, proton T_1 relaxation data of water solutions of the paramagnetic cobalt [5] and manganese [6] carbonic anhydrases showed evidence of exchangeable protons attached to the donor atoms only at high pH values.

The copper derivative, on the contrary, has a donor group with exchangeable protons at every pH between 5.6 and 12 [7,8]. The latter derivative has been suggested to be five coordinate [8]; the monoanionic inhibitors bind at the fifth binding position, the other four donor groups being three nitrogens and a water molecule. Indeed, monoanionic inhibitors do not affect proton T_1 relaxation values [8].

Since we found that several inhibitor derivatives of the cobalt bovine carbonic anhydrase are five coordinate [4] we had to check the presence of water in the donor set together with the three histidyl nitrogens and the inhibitor. It happened therefore to discover that even the pure cobalt enzyme at low pH values shows evidence of exchangeable protons attached to the donor group [9] and that the results [5] were affected by the presence of sulfate ions. At this point both copper and cobalt derivatives have been shown to have a group with exchangeable protons bound to the metal at every pH in the range 5.6–10.

Similar results are obtained for the nickel derivative [10]. Therefore we felt it was important to check the reported relaxivity data of the manganese enzyme [6] before characterization of the active site in metal substituted carbonic anhydrases is attempted.

2. Materials and methods

Bovine carbonic anhydrase was obtained as a lyophilized material from Sigma Chemical Co. All the chemicals used were of the highest commercial purity and all the solutions were prepared using freshly distilled deionized water.

Carbonic anhydrase B was obtained by chromatography on DEAE cellulose as in [11]; only the central part of the first elution peak was collected, which corresponded to about 30% of the eluted protein.

The apoenzyme of both the isoenzyme B and the unpurified material was obtained as in [12] and exhaustively washed against water. Protein concentrations were determined by A_{280}, using a molar absorbance of 57 000 M^{-1} cm^{-1} [13].

^1H longitudinal relaxation times were measured on a Varian CFT 20 spectrometer, operating at 15°C, through the 180°–90° null method. The measurements were performed on both the isoenzyme B and the natural mixture of isoenzymes. The values were found to be the same in both.

3. Results

The manganese–carbonic anhydrase complex is characterized by a relatively low stability constant [6,14], which decreases with decreasing pH values. Therefore free manganese ions and apoenzyme may be present in equilibrium with the metalloenzyme. The solutions for the measurements were prepared by mixing equimolar amounts of manganese sulfate and apocarbonic anhydrase at the unbuffered pH $\simeq$ 6. In fig.1 the proton relaxation rate values are reported for solutions at two different enzyme concentrations.

The values obtained on blank solutions of manganese sulfate at the same concentrations are also reported: the latter data do not show any pH dependence until the drop due to the precipitation of manganese hydroxide.

Relaxation rate values, especially at high pH, are typical of exchangeable protons relaxed through dipolar coupling with a paramagnetic center [15]. The larger values of the enzyme solution with respect to the manganese sulfate solution are due to the difference in the tumbling time [15].

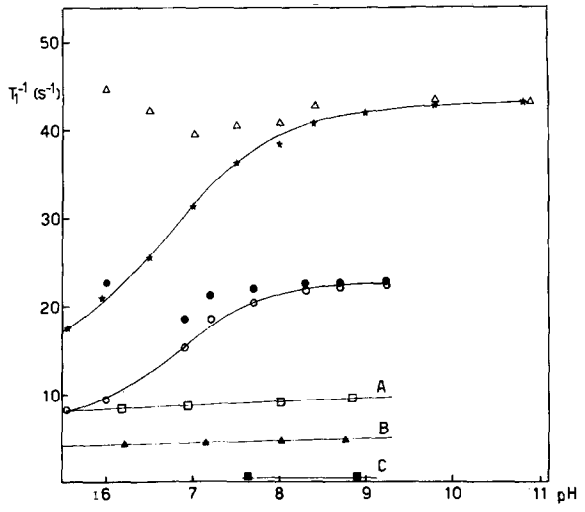

Fig.1. T_1^{-1} values for water protons as a function of pH in solutions containing: (○) Apoenzyme 5 × 10^{-4} M, Mn^{2+} 5 × 10^{-4} M; (★) Apoenzyme 1 × 10^{-3} M, Mn^{2+} 1 × 10^{-3} M; (●,△) T_1^{-1} values obtained from the experimental data, after correction for the dissociation of the manganese enzyme assuming the value of K reported [6]. (A,B) T_1^{-1} values of blank solutions, respectively, 1 × 10^{-3} M and 5 × 10^{-4} M in Mn^{2+}. (C) T_1^{-1} values of pure water.

The more concentrated enzyme solution displays a relaxation time substantially constant in the pH range 11–8, indicating that the acidic and basic forms in equilibrium with pK_a 8.2 [6] have comparable relaxivities. The sigmoid-like curve of fig.1 shifts towards lower pH values with increasing enzyme concentration, in agreement with the presence of a dissociation equilibrium of the metalloenzyme. The relaxation rate of the pure undissociate metalloenzyme has been calculated by taking the used averaged values [6] of the instability constant at various pH values. At high concentration and high pH values the enzyme is almost completely undissociated and, therefore, the effect of the above correction is small. For solutions at low pH values the correction becomes significant: the corrected relaxation values below pH 8 are quite close to the experimental values obtained at high pH values. Thus it is concluded that exchangeable protons are present at every pH.

Measurements [6] on diluted solution containing excess manganese(II) ions agree with the present data at pH > 9 whereas they do not at pH < 8. In the latter mathematical manipulation might have introduced large errors in the calculated relaxation values of the undissociated metalloenzyme due to unfavourable experimental conditions.

Relaxation measurements of manganese(II) carbonic anhydrase were also performed in the presence of increasing amounts of inhibitors at pH 9.0 (fig.2). *p*-Toluene-sulfonamide was found [6] to titrate most of the water relaxivity, while no variation was observed in presence of azide which was reported

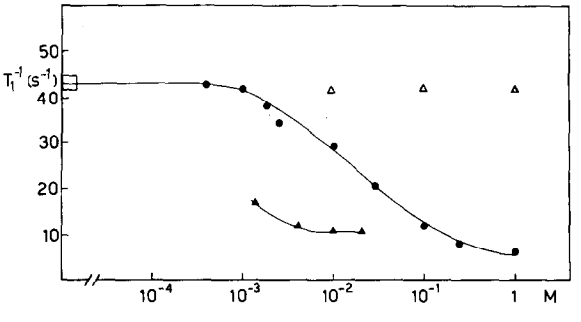

Fig.2. T_1^{-1} values of water protons in a solution containing manganese(II) bovine carbonic anhydrase at pH 9 (□) as a function of the concentration of the inhibitors added: (●) oxalate; (▲) *p*-toluenesulfonamide; (△) azide.

to bind the metal [16]. Interestingly the oxalate ion, which can behave as a bidentate ligand, titrates the relaxation enhancement down to values similar to those obtained with sulfonamides, although only when added in large excess with respect to the manganese enzyme.

4. Discussion

If the manganese ion in the enzyme is assumed to occupy the same site as the zinc ion and to be bound to three histidyl nitrogens [2], the present relaxation data might allow a guess about the coordination number. The azide ion binds to manganese [16] and does not titrate the proton relaxation enhancement; therefore five donor groups have been identified: three histidyl nitrogens, the azide and the group with mobile hydrogens. Although the possibility of six coordination cannot be ruled out with certainty, five coordination seems reasonable. The oxalate ion is bidentate and, as expected, removes the group with exchangeable protons from coordination. The ligand *p*-toluenesulfonamide titrates the relaxation enhancement and presumably behaves as a monodentate ligand, therefore giving rise to a coordination number one unit smaller than with the other inhibitors. In the pure manganese enzyme only four donor groups are identified, therefore the possibility of a larger coordination number cannot be ruled out as ESR data are not of any help in this regard [17]. It should be emphasized that the relaxation data of the manganese enzyme are quite similar to those of the copper analogue [8].

Regarding the nature of the group with exchangeable protons, a single water molecule is consistent with the relaxation values at high pH [6]. The problem is that the same relaxation values are estimated at low pH. If the molecule were water at every pH another group would be responsible for the acid–base equilibrium of the enzyme. Also the cobalt [9], nickel [10] and copper [8] derivatives show constant relaxation within one pH unit from the pK_a of the acid–base equilibrium. Further experimental data and afterthought are necessary; however, the hypothesis of the equilibrium OH_2 –OH^- is consistent with the presence of exchangeable protons attached to a donor group at every pH; the quantitative relation between the relaxation values and the number of protons may be quite complex if other protons, depending on their distance from the metal, contribute to the overall relaxation. The latter contributions have been suggested to be operative in the cobalt [5] and copper [8] enzymes.

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