EVIDENCE OF EXCHANGEABLE PROTONS IN THE DONOR GROUPS OF THE ACIDIC FORM OF COBALT BOVINE CARBONIC ANHYDRASE B

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T_1 relaxation measurements on water protons of solutions containing cobalt(II) bovine carbonic anhydrase have been found to be affected by the paramagnetic center. T_1 shortening has been found to be substantially pH independent. These data are diagnostic of the presence of exchangeable protons in the donor groups of the enzyme, and consistent with a water molecule in the donor set at low pH values. Previous researchers failed to reveal exchangeable protons at low pH values because of the presence of Tris-sulfate buffer which interacts with the metal ion.

Carbonic anhydrase has been shown to have a zinc(II) ion bound to three histidyl nitrogens (1). The overall coordination number has been tentatively suggested to be four for both acidic and basic forms of the enzyme (2) although for the latter a five coordinated structure has also been proposed (3). By analysing the electronic spectra down to 7,000 cm^{-1} of a series of cobalt bovine carbonic anhydrases differing for the inhibiting ligand we believe we have shown that the enzyme itself is four coordinated pseudotetrahedral at every pH in the pH range 5.8-10 (4).

On the basis of X-ray diffraction data at 2.2 A of resolution the fourth ligand has been tentatively proposed to be a water molecule (1). However, proton T_1 relaxation measurements of water solutions containing cobalt(II) bovine carbonic anhydrase in Tris-sulfate buffer were interpreted assuming that no exchangeable protons were present at low pH values (5). Actually proton T_1 values of cobalt(II) bovine carbonic anhydrase in acidic solution containing Tris-sulfate buffer were found to be twice as much the T_1 values at pH 8 with a sygmoidal type of curve. We have reproduced such values. However, if the measurements are performed in absence of buffer, i.e. starting from

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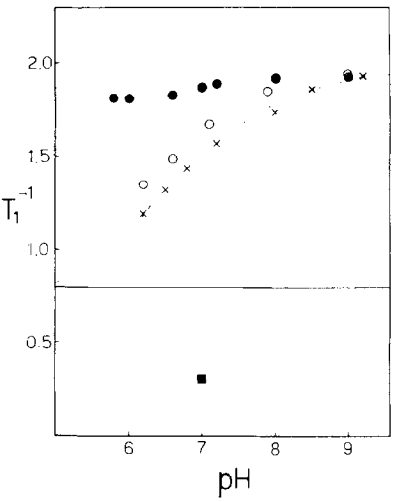

Figure 1. T_1^{-1} values for water protons in a typical enzyme solution as a function of pH: (●) cobalt bovine carbonic anhydrase B, 2.4 x 10^{-3} M; (o) plus Tris-sulfate 10^{-1} M; (x) plus sulfate 10^{-1} M; (———) residual value obtained by adding p-toluenesulfonamide; (■) T_1^{-1} value of H_2O.

pH values near to the isoionic point and adding NaOH to obtain any other pH value, the T_1 values are substantially independent of pH and are typical of the presence of exchangeable protons (5) (Fig. 1). Addition of Tris-sulfate partially titrates T_1 shortening at acidic pH towards the value which has been referred to as "residual relaxivity". Such a value is obtained when the donor group with exchangeable protons is replaced by sulfonamides. Indeed, the simple addition of sulfate ion gives T_1 values whose pH dependence follows the same sygmoidal pattern as in the buffered solution. (Fig. 1). Also the electronic spectra of cobalt(II) bovine carbonic anhydrase B in the visible region are affected by the sulfate ion; in particular the bands at 16,000 cm^{-1} typical of the basic form, which are detectable down to pH 5.8, disappear by adding sodium sulfate to the solution. These results indicate that there is an interaction between the metal ion and the sulfate. Furthermore, one or more anion molecules may bind the protein part in the active cavity (6-9), in such a way that the exchange between protons bound to the donor atom and water protons is slowed down.

These results can be interpreted as a definitive support

to the hypothesys of water being bound to the metal ion in carbonic anhydrase. The substantially constant values of relaxation with pH, although an equilibrium between OH_2 and OH^- is consequential to the model involving coordinated water, can be accounted for by a shorter hydrogen-cobalt distance in the basic hydroxyl containing form with respect to the acidic water containing form.

Cobalt(II) carbonic anhydrase B was prepared from the commercially available enzyme (Sigma Chemical Co.) through the standard procedure (10). The mixture of isoenzymes gives the same relaxation data. ^1H longitudinal relaxation times were measured with a Varian CFT 20 operating at 15 °C.

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