PROTON MAGNETIC RELAXATION IN SOLUTIONS OF MANGANESE-CARBONIC ANHYDRASE

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Received 15 January 1973

1. Introduction

Carbonic anhydrase is a zinc containing metalloenzyme. It catalyzes the reversible hydration of CO_2, as well as the hydrolysis of certain aldehydes (for a review, [1]). It has been shown [1] that the basic form of a group in the enzyme, having a pK near neutral, and being closely linked to the metal ion, is critically involved in the catalysis of the hydration and esterase reactions. However, the identity and the chemical nature of this group is yet unknown. Most investigators claim that a zinc-bound water molecule dissociates above pH 7 to form a zinc-bound hydroxide ion which is enzymatically active [2–5]. Another view is that ionization of an amino acid residue, such as histidine, at the active site, is responsible for the pH dependence of the enzymatic activity [6–8]. In an effort to decide between the two alternative mechanisms we investigated the relaxation rates of the exchangeable water molecules in the manganese coordination sphere of manganese substituted bovine carbonic anhydrase B.

The manganese enzyme caused an appreciable enhancement of the relaxation rates of the solvent water protons at high pH values. This effect was decreased to about 30 ± 5% of its maximum value in the lower pH region, or by addition of sulfonamide inhibitors. Azide and nitrate anions did not affect the relaxation rates. From T_{1p}/T_{2p} ratio we could calculate that the inhibitable part of T_1^{-1}, which is also pH-dependent, is due to one of the water molecules being bound to the manganese ion at the active center. We concluded that the basic form of the group which catalyzes the hydration and esterase reactions is *not* a metal-bound hydroxide ion. Most of our results (except for the anion titration) are qualitatively similar to those obtained by Fabry et al. [9] for proton relaxation rates in aqueous solutions of cobalt (II)–carbonic anhydrase.

2. Materials and methods

Bovine carbonic anhydrase B prepared and purified by the method of Lindskog [10] was obtained from Sigma. Preparation of manganese enzyme from the native zinc enzyme was described in a previous publication [11]. The residual zinc content was determined by atomic absorption (Varian spectrometer type AA-5).

Acetazolamine and p-toluenesulfonamide, obtained from Koch-Light Laboratories, were used without further purification.

Longitudinal relaxation times, at 5–60 MHz, were measured with a Bruker type B-KR-322 pulsed spectrometer and at 100 MHz using a spin echo attachment to high resolution NMR spectrometer [12], by the 180°–90° null method. Values of T_2 were obtained at 100 MHz from the spectral linewidth, using the expression $1/T_2 = \pi\Delta\nu$, where $\Delta\nu$ is the full line-width at half maximum peak height.

3. Results

Fig. 1 shows the pH dependence, at 60 and 100 MHz, of the paramagnetic contribution to the longitudinal relaxation rate ($1/T_{1p}$) of water protons in aqueous solutions of Mn(II)–carbonic anhydrase. The relaxation rates were corrected using the stability constants of manganese (II)–bovine carbonic anhydrase complex at each pH value (Lanir and Navon, to be

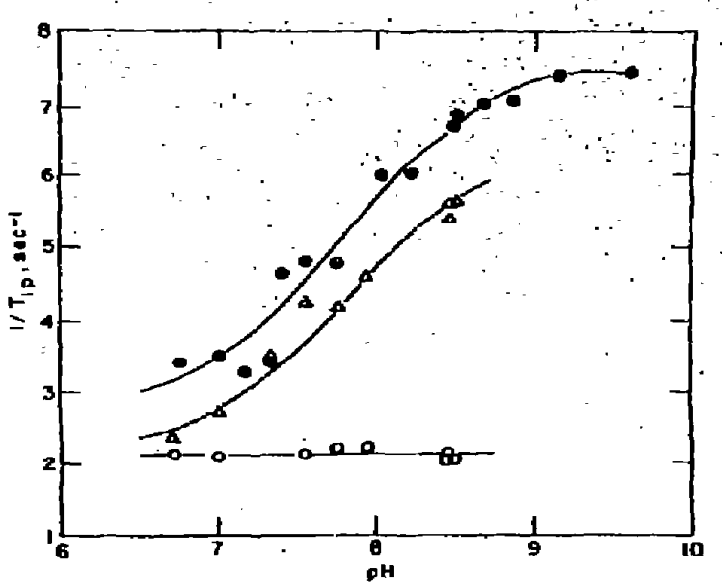

Fig. 1. The pH dependence of the longitudinal relaxation rate for 1.9×10^{-4} M bovine manganese (II)–carbonic anhydrase in 0.05 M Tris-sulfate buffer. The solutions contained 2.5×10^{-4} M $MnCl_2$. The relaxation rates were corrected according to the Mn-(II)–enzyme stability constants at each pH value. • = Relaxation rates at 60 MHz. △ = Relaxation rates at 100 MHz. ○ = Relaxation rates at 100 MHz of p-toluenesulfonamide-inhibited manganese enzyme.

published). The data could be fitted with a pK value of 7.8 ± 0.2. This value is somewhat higher than the apparent pK of 7.0–7.5 for the zinc and cobalt bovine enzymes, obtained by activity measurements and spectral titrations [6, 13–16].

Fig. 2 presents the effect of anions and the sulfonamide inhibitor acetazolamide on the relaxation rate, $1/T_{1p}$, of the water protons. While addition of acetazolamide resulted in a great decrease in the relaxation rate, the addition of both N_3^- and NO_3^- ions did not affect the relaxation rate. Similar titration was observed with p-toluenesulfonamide. The residual relaxivity in its presence amounted to 30 ± 5% and was found to be pH independent (fig. 1). Furthermore, the relaxivity extrapolated to low pH values coincides with the relativity in the presence of the sulfonamide inhibitors.

The net proton relaxation rates for solutions of manganese containing complexes, are given by the expressions:

$$\frac{1}{T_{ip}} = \frac{Nq}{55.6} \frac{1}{T_{iM} + \tau_M}; \quad i = 1,2.$$

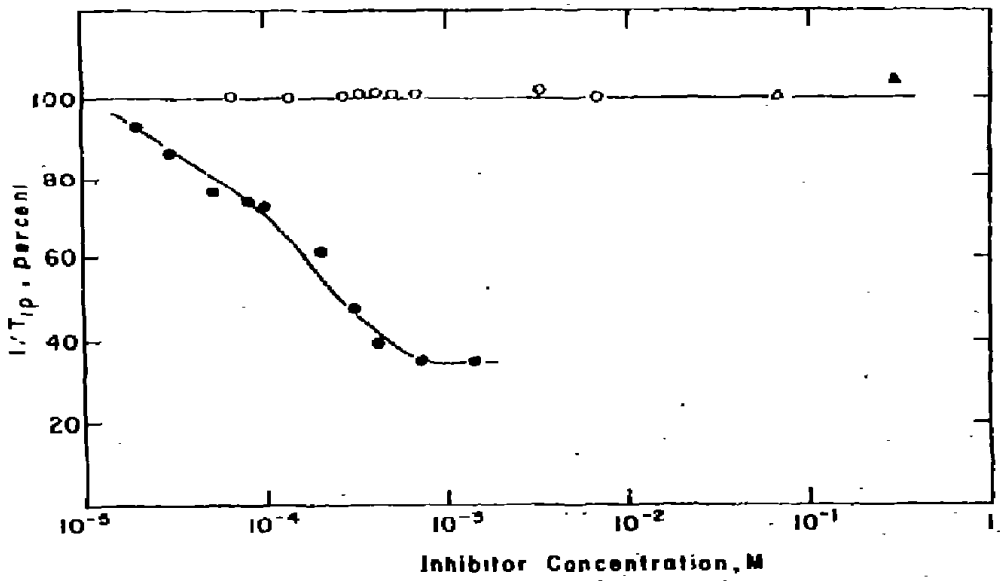

Fig. 2. Percent relaxivity plotted against inhibitor concentrations. The effect of anions was studied at 60 MHz, 25°, in solutions of 2.2×10^{-4} M Mn(II)–enzyme, containing 5×10^{-4} M $MnCl_2$. ○ = Azide ions in 0.01 M Tris-sulfate buffer pH = 7.86. △ = Azide ions in 0.01 M Tris-sulfate buffer pH = 7.32. ▲ = NO_3^- ions in 0.06 M Tris-sulfate buffer pH = 8.4. • = Titration of 1.9×10^{-4} M Mn(II)–carbonic anhydrase by acetazolamide at 100 MHz, 30°. The solutions contained 1.3×10^{-4} M $MnCl_2$. 0.026 M Tris-sulfate buffer pH = 8.5.

In our case, N is the concentration of the Mn(II)–carbonic anhydrase; q, its hydration number; T_{1M} and T_{2M}, the relaxation times of the water protons in the first coordination sphere, and τ_M the residence time of the nucleus in the first coordination sphere.

In a series of experiments, it was found that T_{1p} is frequency dependent, in the range 5–100 MHz. Moreover, T_{2p} at 100 MHz was found to increase with temperature. The T_{1p}/T_{2p} ratio was found to be 3.5 at 100 MHz and 30°. All these results strongly indicate that the condition of fast exchange holds for the present system. In such condition, the hydration number of the manganese, in the active site of a manganese containing enzyme is given by [17]:

$$q = 3.26 \times 10^{-14} \frac{(T_{1p}/T_{2p}) - 0.5}{[(T_{1p}/T_{2p}) - 1.19]^{1/2}} \cdot \frac{\omega_I}{NT_{1p}}$$

where ω_I is the angular frequency for the nuclear spin. Using this expression, it was calculated that 0.98 ± 0.05 water molecules are displaced by sulfonamide inhibitor, and 0.96 ± 0.05 water molecules are titratable within the pH range of 9.0 to 6.7.

The correlation time for the manganese–proton interaction can be calculated from the experimental T_{1p}/T_{2p} ratio [17] and was found to be 3.0×10^{-9} sec at 100 MHz and 30°. An important result is that the ratio T_{1p}/T_{2p}, and hence the correlation time, did not vary upon either titration with sulfonamide inhibitors or pH variations. This means that the above titrations involve a displacement of one water molecule and not a change in the relaxation mechanism. Furthermore it suggests that the residual relaxation rate is not due to residual slow exchanging water molecules in the manganese coordination sphere. It may originate from water molecules in the second coordination sphere or also from fast exchanging protons on the ligands of manganese atom at the active site.

4. Discussion

The mechanism of catalysis of the reversible hydration of CO_2 by carbonic anhydrase is not yet known with any certainty. A variety of studies have indicated that the ionization of a group with a pK near 7 is the basis of the pH dependence of the activity. Most investigators favor the interpretation that the catalysis is intimately dependent on a metal bound water molecule, which can dissociate above pH 7 to give the active form ($ZnOH^+$ in that case) in the hydration reaction. However, the immediate conclusion from the relaxation rate measurements of solvent water protons of Mn(II)–carbonic anhydrase, is that one water molecule (and not a hydroxide ion) is directly linked to the metal ion at high pH values, while probably no fast exchanging water molecule is liganded to the metal ion at low pH values. It seems, therefore, that at high pH values, an ionization on the protein moiety is responsible for the activation of the enzyme. Similar conclusions were recently suggested by Koenig and Brown [8] to explain their proton relaxation rates measurements in cobalt–(II) carbonic anhydrase solutions [9].

Fig. 2 presents the titration of a single water molecule from the coordination sphere of the metal ion by a sulfonamide inhibitor. This result is compatible with the X-ray data [18, 19] for direct binding of the sulfonamide to the metal ion.

Azide ions are known to bind at or near the active site of manganese (II)–carbonic anhydrase, with an equilibrium constant of about 3×10^{-4} M at pH = 7.5, as was demonstrated by competition experiments with sulfacetamide [11] and acetate ions (Lanir and Navon, to be published). In the present work it is evident (fig. 2) that azide and nitrate anions had no effect on the water molecules bound to the manganese enzyme, even when the azide concentration was two orders of magnitude higher than its dissociation constant. These results seem to question the direct binding of anions to the manganese ion. Other alternatives are that binding of the anion increases the coordination of the metal, or that the anion replaces one of the metal ligands.

We have repeated and confirmed the result of Fabry et al. [9] that anions do replace the water molecule bound in the first coordination sphere of the cobalt ion in Co (II)–carbonic anhydrase. Thus, the binding mode of anions may be different for the cobalt and manganese carbonic anhydrases. However, the two metalloenzymes are similar in that they have a single water molecule bound to the metal ions in the basic forms of the enzymes which is absent in their acidic forms.

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