A ^{31}P NMR STUDY OF PHOSPHATE IN PRESENCE OF COBALT(II)- AND COPPER(II)-SUBSTITUTED BOVINE CARBONIC ANHYDRASE B

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1. Introduction

Carbonic anhydrase is a zinc-containing metalloenzyme which catalyzes the reversible hydration of carbon dioxide [1–3]. Its extremely high catalytic efficiency is still a challenge to those researchers who have postulated suitable mechanisms for its action; among many proposals, recently a buffer-mediated proton transfer has been hypothesized as a step in the catalytic pathway [4]. Indeed, the enzyme operates both in vivo and in vitro in the presence of buffering species and most of the studies of the enzyme are performed in presence of various buffers. Therefore the interference of the buffering species with the various experiments deserves some attention [5]. For instance the Tris–sulphate buffer has been found to reduce the proton relaxation enhancement of water molecules due to the presence of cobalt carbonic anhydrase [6]. Furthermore the sulphate as well as the phosphate gives rise to an electronic spectrum of the cobalt enzyme very similar to that of the pure acidic form of the metallo enzyme [7,8]. The problem remains whether the effect of the sulphate ion is to directly bind the cobalt or to reflect a change in the ionic strength.

In order to gain more direct information on the influence of buffering species we have investigated by means of ^{31}P NMR spectroscopy the interaction between the phosphate ions and carbonic anhydrase in which the native zinc ion is substituted by cobalt(II) or copper(II). Only the former paramagnetic ion gives rise to a still active enzyme [3].

2. Materials and methods

Bovine carbonic anhydrase (BCA) was obtained by Sigma Chemical Co. and purified by chromatography on DEAE cellulose to obtain the isoenzyme B, which was treated as in [3] to obtain zinc-free apoenzyme. The apoenzyme was dialyzed against 2.0×10^{-1} M phosphate buffer at pH 6.2, concentrated by ultradialysis $\leqslant 10^{-3}$ M and finally reacted with slightly less than the stoichiometric amount of cobalt or copper sulphate solutions. The metal content was tested through electronic spectroscopy, by comparing the molar absorbance of the main d–d transition of each metal derivative with the absorption band of the protein. Some apoprotein was also reacted with zinc sulphate to give the reconstituted native enzyme. The pH was raised from 6.2 up to 9–10 by direct addition of NaOH.

^{31}P NMR spectra were recorded with a Varian CFT20 spectrometer operating at 10°C, unless otherwise specified. The samples contained D_2O for the lock signal. T_2 values were obtained through the relation $T_2 = (\pi\Delta\nu)^{-1}$ where $\Delta\nu$ is the linewidth at half peak height measured without applying any weighting function to the free induction decay. T_1 values were obtained through the inversion recovery method. ^{1}H relaxation measurements were performed as in [7].

3. Results and discussion

3.1. *Metal binding*

The presence of paramagnetic metal ion in the active site of carbonic anhydrase influences T_1 and T_2 values of the ^{31}P signal (see table 1). Such effect is orders of magnitude larger than that caused by the native diamagnetic enzyme ZnBCAB. T_2 is always shorter than T_1, either because the former time reflects contact contributions in the limit of fast exchange or because it is influenced by the ligand residence time [9]. Measurements at various temperatures (5–35°C) may shed light on the problem. For the cobalt derivative T_1 maintains the same value as reported in table 1, whereas T_2 decreases with increasing temperature. This pattern is indicative that the phosphate exchange rate is fast only with respect to T_1. In the case of the copper derivative also T_1, besides T_2, decreases with increasing temperature, indicating that both the relaxation times are mostly affected by the residence time of the ligand in the paramagnetic site. The difference between T_2 and T_1 for both cobalt and copper derivatives as well as the difference in their temperature dependence in the former case indicates that the resonating nuclei experience some isotropic shift which, however, cannot be determined since the NMR signal is not on an averaged position. The occurrence of isotropic shift is a proof of phosphate–enzyme binding close to the metal.

The specificity of this interaction is confirmed by titration of the paramagnetic effect by means of cyanate, a strong inhibitor of CoBCA [10]. In fig.1

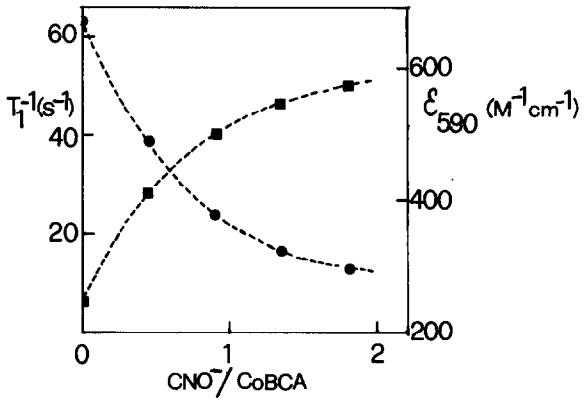

Fig.1. Titration of ^{31}P T_1^{-1} enhancement in presence of CoBCAB with cyanate (•) and parallel increase of molar absorbance of the CoBCAB–cyanate adduct (▪) (conditions as in table 1).

the decrease of ^{31}P T_1^{-1} is shown together with the parallel increase of $A_{590\ nm}$ in the electronic spectrum of the cobalt enzyme due to the formation of the cyanate adduct. As more than 85% of the limiting values of both molar absorbance and ^{31}P T_1^{-1} are attained with a cyanate–enzyme ratio of 1.3/1 it follows that the phosphate binds to the enzyme in a 1/1 ratio. In fig.2 the decrease of the water proton relaxivity by addition of the phosphate buffer to an unbuffered solution of CoBCAB is shown: probably water is removed from coordination upon phosphate binding.

In principle T_1 values can be used to calculate metal–nucleus distances through the Solomon-

Table 1
^{31}P T_1 and T_2 values of phosphate 1.8×10^{-1} M, at pH 6.2 and 10°C in presence of various metallo-substituted carbonic anhydrases

	T_1 (s)	T_2 (s)
CoBCAB 9.1×10^{-4} M	2.0×10^{-2}	1.6×10^{-3}
CuBCAB 8.7×10^{-4} M	1.1×10^{-2}	7.2×10^{-3}
ZnBCAB 9.1×10^{-4} M	9.4×10^{-1}	1.0×10^{-1}

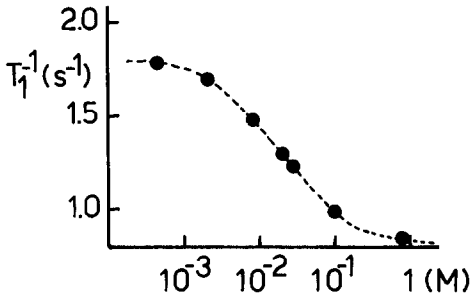

Fig.2. Decrease of T_1^{-1} enhancement of water protons in presence of CoBCAB 2.4×10^{-3} M increasing amounts of phosphate buffer, pH 6.

Bloenbergen equation [11], if the condition of fast exchange is met, as in the case of the cobalt derivative; however, severe limitations to the reliability of such calculations arise from several factors:

(i) Possibility of large anisotropy in the magnetic tensor;
(ii) Uncertainty on the values of the correlation times τ_c to be used as parameters;
(iii) Possibility of delocalization of unpaired spin density onto non-s orbitals of the resonating nucleus [12].

Attempts to calculate the Co–P distance via the above equation using the value for τ_c of 1×10^{-11} s as determined [13] provide a value $\simeq$200 pm which is too short for any acceptable Co–O–P angle; neither a different choice of τ_c within a range of reasonable values (10^{-12}–5×10^{-11} s) brings the distance to fit any structural model. Presumably in this case the effect of a 'ligand-centered' dipolar relaxation mechanism is sizeable [12,14]. Although there is not any direct proof of contact interactions, the change of the electronic spectra due to phosphate addition [8] and the hypothesis of a ligand-centered mechanism due to spin delocalization leads to the conclusion of a direct phosphate–metal binding.

3.2. *Influence of pH*

Carbonic anhydrase is known to undergo an acid–base equilibrium which is linked to its catalytic properties; it has been shown that such equilibrium involves the metal ion environment, since the electronic spectra of CoBCA are pH dependent [1,2,7]. Most of the inhibitors bind the acidic form of the enzyme. The pK_a for this equilibrium is around 7 for zinc and cobalt derivatives [1] and 8 for the copper derivative [5]. On the other hand the $H_2PO_4^-$ undergoes deprotonation with pK_a 7.2, which, however, can be somewhat shifted towards lower values in concentrated solutions as in the present experiment. In order to gain information on the species involved in the phosphate–metal binding, ^{31}P relaxation measurements were performed as a function of pH values. As shown in fig.3 the ^{31}P T_1^{-1} values decrease with increasing pH, indicating that the apparent affinity is larger at low pH values. However, the decrease is not as sharp as it would be expected if binding occurred only between the acidic form of the enzyme EH^+ and the acidic species of the buffer $H_2PO_4^-$. This suggests that some additional interactions between the acidic form of the enzyme and the HPO_4^{2-} ion or between the basic form of the enzyme and $H_2PO_4^-$ could be operative. Further investigations of these additional interactions would require an evaluation of the activity coefficients in such concentrated solutions and probably a different approach based on potentiometric titrations.

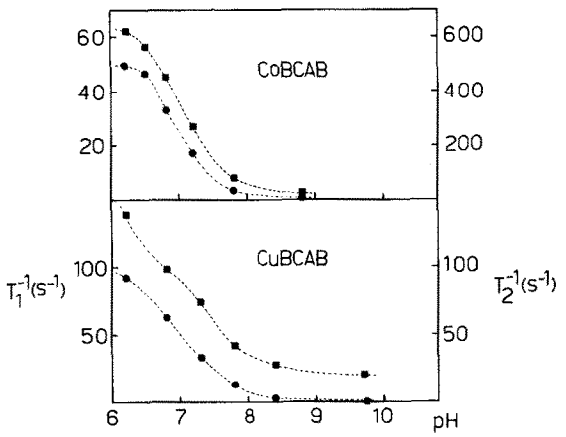

Fig.3. pH dependence of T_1^{-1} (•) and T_2^{-1} (▪) of phosphate ^{31}P in presence of CoBCAB and CuBCAB (conditions as in table 1).

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