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Characterization of Oxovanadium(IV) Substituted Bovine Carbonic Anhydrase B

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The compound obtained by combining the oxovanadium(IV) ion and apocarbonic anhydrase in water has been further characterized by means of electronic and nmr spectroscopy. The electronic spectra are typical of tetragonal complexes whereas water proton T_1 values indicate that a water molecule is bound to the paramagnetic center.

The coordinating capability of the active cavity with respect to the various metal ions is comparatively discussed.

Introduction

Carbonic Anhydrase is a metalloenzyme in which the zinc(II) ion is bound to the protein part through three histidine nitrogens [1]. The X-ray data at pH 8.5 [1] have shown that a fourth small ligand completes the coordination polyhedron with a pseudotetrahedral arrangement. The enzyme displays an acid–base equilibrium apparently with a single pK_a value between 6.6 and 7.3 depending on the origin of the enzyme [2]. One of the major problems in the current debate on Carbonic Anhydrase is the characterization of the fourth ligand in the coordination sphere and of its role, if any, in the acid–base equilibrium. A reasonable candidate for such ligand is a solvent molecule [3].

The native zinc(II) ion can be removed without definitive alteration of the tertiary structure of the enzyme and can be substituted by other bipositive ions [4]. Among the metal substituted enzymes the cobalt derivative shows an enzymatic activity comparable to that of the native enzyme, as well as an acid–base equilibrium [4]. Since the cobalt ion is paramagnetic with three unpaired electrons, if water is bound to the metal and exchanges rapidly on the nmr time scale, direct information can be obtained from the nmr spectra of the solvent [5]. In particular water proton T_1^{-1} values of solutions containing the cobalt enzyme are indicative of the presence of a group with exchangeable protons bound to the metal at every pH between 6 and 10 [6]. Unfortunately there is no theoretical tool that in the present case will allow to determine the number of exchangeable protons, since the parameters determining the relaxing capability are not known; however, the investigation of manganese [7] and copper [8] derivatives have allowed to reasonably support the hypothesis of a water molecule bound to the metal. Since the oxovanadium(IV) ion carries an oxygen as a further ligand, we have investigated its enzyme derivative in order to check whether water is still present and to obtain information on the coordination versatility of the active cavity.

Experimental

Bovine Carbonic Anhydrase (EC 4.2.1.1.) was obtained from Sigma Chemical Company and chromatographed with DEAE Cellulose to obtain the B isoenzyme [9]. Oxovanadium(IV) sulfate and all the other chemicals used were of analytical grade; all solutions were prepared using freshly bidistilled water. Zinc removal was obtained through dialysis against solutions of 2,6-piridine dicarboxylic acid 5×10^{-2} *M*, in phosphate buffer 2×10^{-1} *M* pH 6.9, according to a recent report [10]. The apoenzyme solution was exhaustively dialyzed against bidistilled water, concentrated by ultradialysis, degassed and reacted with degassed oxovanadium(IV) sulfate solutions in slightly less than the stoichiometric amount. The pH of the samples was varied by addition of NaOH.

The electronic spectra were run on a Cary 17D spectrophotometer within few hours from the VO derivative preparation using unbuffered oxovanadium(IV)–enzyme (VOBCAB) solutions 2–3 $\times$ 10^{-3} *M*, in the absorption range 0–0.1. Aqueous solutions of inhibitors were added directly in the spectrophotometric cells using micropipettes.

Water proton relaxation measurements were performed on a VARIAN CFT 20 NMR operating at 15 °C using freshly prepared 3.5×10^{-3} *M* VOBCA solutions. ^{13}C NMR measurements were performed on the same spectrometer, using 20% D_2O as internal lock. T_1 values were obtained using the inversion

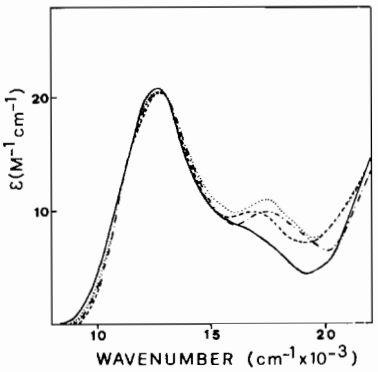


Fig. 1. Electronic spectra of oxovanadium(IV)–bovine carbonic anhydrase B(———); plus 1:1 molar ratio of NCS^- (-------); I^- (–·–·–); N_3^- (.....).

recovery method, with the experimental procedure described elsewhere [6]. The reproducibility of the reported values for several measurements and different enzyme concentrations 2–4 × 10^{-3} *M* is within ±5%.

Results

The electronic spectra (Fig. 1) measured at pH values between 6 and 7.5 show two main bands at 12,900 and 17,200 cm^{-1}. The spectrum is pH independent up to pH values of about 8 and then the absorption bands decrease in intensity presumably as a result of decomposition.

Addition of most of the inhibitors of the native enzyme does not alter the electronic spectrum; in the case of iodide, azide, and thiocyanate the band at *ca.* 17,000 cm^{-1} slightly increases in intensity and is better resolved.

The esr spectra have been already reported [11]. They show the typical octet due to the hyperfine splitting with $A_\parallel = 90.6 \times 10^{-4}$ cm^{-1}. At pH > 8 the spectrum dramatically broadens. Inhibitors do not affect the esr spectrum.

The water proton T_1^{-1} values of a solution containing the oxovanadium(IV) enzyme 3.5 × 10^{-3} *M* are shown in Fig. 2. The T_1^{-1} enhancement is significantly large with respect to the values of diamagnetic solutions indicating that exchangeable protons are attached to the metal. Increasing addition of inhibitors up to 3:1 ratios does not alter the T_1^{-1} values. In the case of N_3^-, a large excess causes a decrease in T_1^{-1} enhancement. In order to check the nature of the interaction between the inhibitor and the metalloenzyme a ^{13}C T_1 study on $N^{13}CS$ has been undertaken. The ^{13}C longitudinal relaxation time for a solution 2.2 × 10^{-1} *M* of $KN^{13}CS$ in the presence of VOBCAB 2.2 × 10^{-3} *M* was found to be 0.36 s which compares with 10 s for a solution containing the pure thiocyanate ion. Again for comparison purposes the

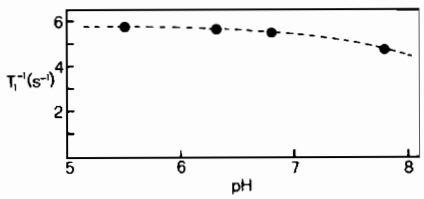


Fig. 2. pH dependence of T_1^{-1} values of the ^1H signal of unbuffered water solutions containing 3.5 × 10^{-3} *M* oxovanadium(IV)–bovine carbonic anhydrase.

relaxation time of the same inhibitor bound to the cobalt derivative of carbonic anhydrase under the same concentrations was measured. The ^{13}C relaxation time was found to be more than 10 times shorter, although the oxovanadium(IV) ion has a larger relaxing capability than the cobalt(II) ion (*vide infra*). This shows that in the case of VOBCA the thiocyanate ion feels the paramagnetic center but at a distance definitely larger than that allowed by any binding interaction.

Discussion

The T_1^{-1} enhancement of water protons in principle provides information on the number of exchangeable protons bound to the coordination polyhedron through the Solomon–Bloembergen equation [12]:

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{S(S+1)\gamma_I^2 g^2 \beta^2}{r^6} f(\tau_c) \qquad (1)$$

with

$$f(\tau_c) = \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}$$

where τ_c is the correlation time, T_{1M}^{-1} is the paramagnetic contribution to T_1^{-1} calculated for the pure adduct, r is the distance between the proton and the paramagnetic center and the other symbols have the usual meanings. This relationship holds within the frame of an isotropic magnetic tensor and of a dipolar relaxation mechanism, the unpaired electrons being located at the metal. Unpaired electrons delocalized onto the ligand are a large source of error which actually prevents from determining the number of protons [13]. However, the comparison between the T_1^{-1} enhancements of the various metal carbonic anhydrases may be meaningful. In Fig. 3 the dependence of $f(\tau_c)$ on τ_c is reported. The curve shows a maximum between 10^{-8} and 10^{-9}s. The $f(\tau_c)$ values may also be calculated from the experimental T_1^{-1} enhancements of the various metal derivatives [6–8, 14], by assuming that a single water molecule is

TABLE I. Optical Absorption Spectra of Some Representative VO^{2+} Complexes.

Complexes	Coordination Number	Donor Set	Frequencies ($cm^{-1} \times 10^{-3}$)	
			ν_1	ν_2
$VO(H_2O)_5$[a]	6	O_6	13.0	16.0
$VO(acac)_2$[b]	5	O_5	15.2	16.8
$VO(NCS)_5$[c]	6	ON_5	13.7	17.8
$VO(Cl)_5$[d]	6	OCl_5	15.5	16.4
$VO(CN)_5$[c]	6	OC_5	15.6	24.8
VO(SALen)[e]	5	O_3N_2	16.0	17.0
VO Insulin[f]	6	O_6	12.9	16.7
VOBCAB[g]	–	–	12.9	17.2

[a]C. J. Ballhausen and H. B. Gray, *Inorg. Chem., 1,* 111 (1962). [b]J. Bernal and P. H. Rieger, *Inorg. Chem., 2,* 256 (1963) (acac = acetylacetonate). [c]J. R. Wasson, *J. Inorg. Nucl. Chem., 20,* 171 (1968). [d]R. A. D. Wentworth and T. S. Piper, *J. Chem. Phys., 41,* 3884 (1964). [e]L. J. Boucher, E. C. Tynan, and T. F. Yen, *Inorg. Chem., 8,* 689 (1969); (SALen = bis(salicylaldehyde)ethylenediimine). [f]M. D. Chasteen, R. J. DeKoch, B. L. Rogers, and M. W. Hanna, *J. Am. Chem. Soc., 95,* 1301 (1973). [g]This work.

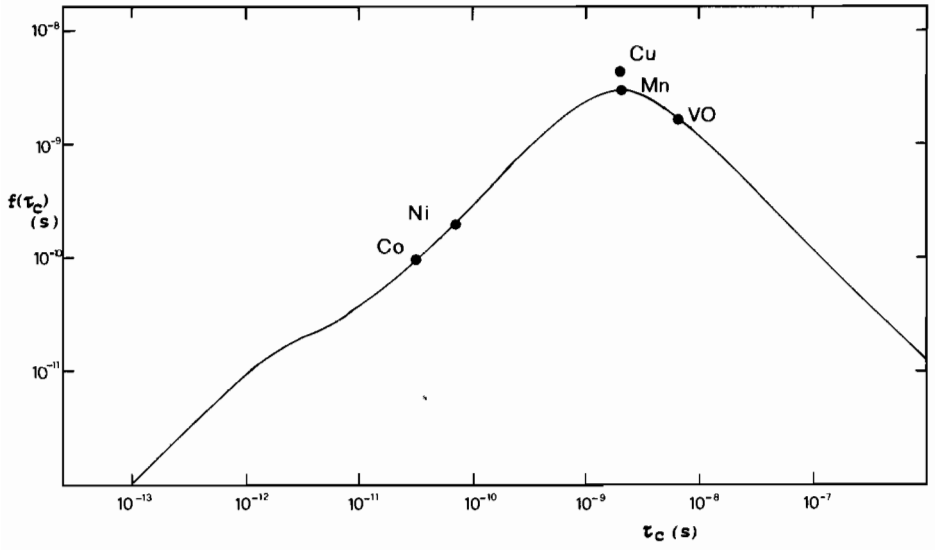


Fig. 3. $f(\tau_c)$ part of the Solomon–Bloembergen equation (1) as a function of τ_c. The dots represent the $f(\tau_c)$ values calculated through equation (1) using the relaxation data for cobalt, nickel, copper, manganese, and oxovanadium(IV) BCAB derivatives and taking r = 2.8 A.

bound to every metal, except Ni^{2+} which is assumed to be bound to two water molecules. If these values are reported in the same plot on the $f(\tau_c)$ line, and if it is kept in mind that the maximum value of τ_c is that of the macromolecule rotation ($\cong 10^{-8}$ s), the experimental τ_c may be estimated. They seem to follow the order

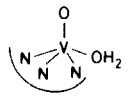
$$Co^{2+} < Ni^{2+} < Mn^{2+} \cong Cu^{2+} < VO^{2+}$$

which actually is the expected order for electronic relaxation times on the ground of experimental esr linewidths [8, 15]. These considerations support the assumption that a water molecule is retained within the coordination sphere also in the case of the oxovanadium(IV) enzyme derivative.

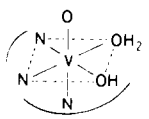
The electronic spectra show two main absorptions which are typical of most VOL_4 and VOL_5 chromophores [16, 17]. They are reasonably assigned within a C_{4v} idealized symmetry as $d_{xy} \rightarrow d_{xz}, d_{yz}$ and $d_{xy} \rightarrow d_{x^2-y^2}$ respectively [18]. The energies of these transitions, however, are not much affected on passing from five to six coordination (Table I): presumably a sixth ligand *trans* to the oxygen causes a small perturbation on the energy levels and in particular a shift towards high energies [17].

Conceivably the oxovanadium(IV) ion, analogously to any other metallo-derivatives, retains three

histidine nitrogens as donor atoms besides the water molecule revealed by the relaxation measurements. Since the z axis of the C_{4v} point group is given by the V–O direction and since the three histidine nitrogens cannot be coplanar with the metal, the resulting geometry should be a somewhat distorted square pyramid. The resulting N_3O_2 chromophore would reasonably be expected to show electronic absorptions intermediate between the chromophores $VO(H_2O)_5$ and $VO(NCS)_5$.



A recent X-ray report [19] on the compound obtained from the apocarbonic anhydrase and $HgCl_2$ has shown that the hydroxy group of threonine 197, which in the native enzyme is 4 Å from the zinc ion and hydrogen bonded to the water ligand, can directly bind the metal in the active site. In fact the mercury atom reaches six coordination through the three histidine nitrogens, the threonine hydroxy group, and the two chlorines, which are *cis* to each other. Binding of the threonine residue requires some concomitant displacement of the metal (of about 0.8 Å from the position of the zinc) and of histidine 93. Therefore the possibility cannot be ruled out that the oxovanadium(IV) ion also is hexacoordinated through threonine binding.



Such a group is also a reasonable candidate as the sixth ligand in the case of the nickel enzyme [14], together with two water molecules. Finally, in the case of the copper enzyme a five coordination of the chromophore was suggested [8] with the fifth ligand, which could as well be this threonine residue, at a distance larger than usual, the other donor groups being the same three histidine nitrogens and a water molecule.

The present data definitely suggest the cavity to be shaped in such a way that it may contain two donor groups contemporarily bound to the metal: such groups in the present case are the oxygen of the oxovanadium(IV) and a water molecule, two water molecules in the case of the nickel(II) derivative [14], and in the case of five coordinated inhibited cobalt [6, 20] and copper [8] enzyme a water molecule and the inhibitor.

Acknowledgments

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Note added in proof:

Discussion with Professor E. Clementi, Donegani Institute Novara, who performed Monte Carlo calculations on carbonic anhydrase [21], confirmed to us that the active site of carbonic anhydrase is able to accomodate two later molecules in the inner coordination sphere of the metal.

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