Biophysical Chemistry 8 (1978) 267–270

AN ELECTRON SPIN RESONANCE STUDY OF HIGH SPIN FORMS OF COBALT(II) BOVINE CARBONIC ANHYDRASE

Alessandro DESIDERI[1], Laura MORPURGO, J. Barrie RAYNOR[2] and Giuseppe ROTILIO
Institute of Biological Chemistry and CNR Center of Molecular Biology, University of Rome, Rome, Italy

Received 16 December 1978

The ESR spectra of bovine Co(II) carbonic anhydrase at 7 K at low and high pH and of the iodide derivative have been analyzed. The spectrum of the low pH form shows axial symmetry whilst that at high pH is rhombically distorted. This anisotropy is still more accentuated in the iodide derivative. The high pH (hydroxyl) form and the iodide derivative are thought to have a tetracoordinate trigonal pyramidal structure, with a fifth more distant axial ligand. The low pH form is consistent with a pseudotetrahedral geometry previously postulated.

1. Introduction

Bovine carbonic anhydrase (BCA) is a monomeric zinc(II) containing enzyme, molecular weight 29000, which catalyzes the reversible hydration of CO_2 to H_2CO_3 [1,2]. Cobalt (II) is the only paramagnetic ion to substitute for zinc and to retain substantially the same activity as the parent enzyme [3].

Many physical and spectroscopic measurements have been carried out in order to elucidate the bonding and the stereochemistry at the metal site. This has led to the hypothesis that Co(II) has pseudotetrahedral or pentacoordinate symmetry [2,4]. A recent study of the optical spectra suggests a substancially tetrahedral structure for both the low and the high pH forms and an equilibrium between tetra and pentacoordination for the halide derivatives [5]. To gain more information about this analysis, we have investigated the ESR spectra of the iodo derivative and of the low and high pH forms of Co(II) BCA.

2. Materials and methods

BCA was prepared as previously described [6]. After removal of Zn(II) by dialysis against 10^{-3}M *o*-phenanthroline in 0.1 M acetate buffer pH 5.2, Co(II) was added in slightly less than stoichiometric amount in the form of 0.1 M $CoSO_4$. The iodo form was prepared by adding 0.1 M NaI to 0.9 M Co(II) BCA in water. The hydroxyl form was prepared by adding concentrated NaOH solution to Co(II) BCA until the pH was 9.5.

X-band ESR spectra were recorded with an E-9 Varian spectrometer. Temperature control was obtained with an Air Products and Chemicals CT-3-110 liquid-transfer Cryo-Tip refrigerator with automatic temperature controller. pH measurements were obtained with a Radiometer pHM 63 Digital pH-meter.

3. Theory

The free Co(II) ion has a ^4F ground state which in a trigonal bipyramidal field is split into five levels (fig. 1). The $^4A_2'$ ground state mixes via spin orbit coupling with the excited $^4E''$ level forming the two Kramers doublets $m_s = \pm 1/2$ and $m_s = \pm 3/2$ separated by the energy $\delta = 2(D^2 + 3E^2)^{1/2}$ (the zero field splitting). If the zero field splitting is too large for the micro-

---

[1] Present address: Department of Physics, University of Calabria, Arcavacata, Italy.

[2] Permanent address: Department of Chemistry, University of Leicester, Leicester, U.K.

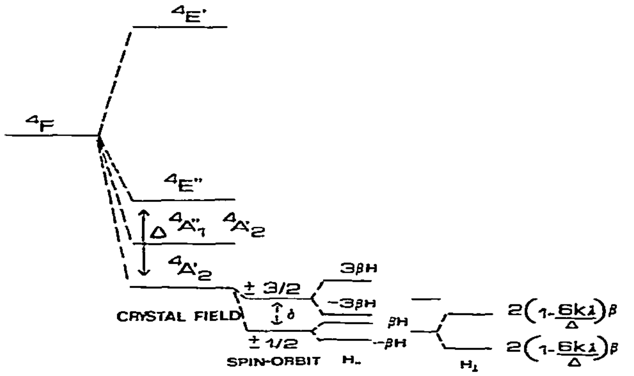

Fig. 1. Energy level diagram of the Co(II) ion in a trigonal bipyramidal crystal field.

wave frequency used, as is generally the case, only the transition between $|-\frac{1}{2}\rangle$ and $|+\frac{1}{2}\rangle$ is possible. In this case we can use two approximations: either $S' = \frac{1}{2}$ and $m_s = \pm\frac{1}{2}$ only, or $S = \frac{3}{2}$ and the secular determinant is diagonalized within the $m_s = \pm\frac{1}{2}$ doublet using wavefunctions which are linear combinations of $m_s = \pm\frac{1}{2}$ and $m_s = \pm\frac{3}{2}$; if necessary. In the case of axial symmetry $E = 0$ and the relationships between the intrinsic values g_x, g_y and g_z in the $S = \frac{3}{2}$ approximation and the principal values g'_x, g'_y and g'_z in the $S' = \frac{1}{2}$ case are:

$$g_z = g'_z; \qquad g_x = g_y = \tfrac{1}{2}\, g'_x = \tfrac{1}{2}\, g'_y. \tag{1}$$

For rhombic symmetry $E \neq 0$ and the spin states $m_s = \pm\frac{1}{2}$ mix with the spin states $m_s = \pm\frac{3}{2}$ and the ground state is depicted by the wavefunctions

$$\psi_1 = \alpha|\tfrac{3}{2}\rangle + \beta|-\tfrac{1}{2}\rangle$$

$$\psi_2 = \alpha|-\tfrac{3}{2}\rangle + \beta|\tfrac{1}{2}\rangle \tag{2}$$

where the coefficients α and β are such that $\alpha^2 + \beta^2 = 1$ and their value depends on the ratio between the rhombic and axial component of the crystal field $\gamma = E/D$, which itself is linked to β by

$$\beta^2 = (2 + 3\gamma^2 + 2\sqrt{1 + 3\gamma^2})/(2 + 6\gamma^2 + 2\sqrt{1 + 3\gamma^2}) \tag{3}$$

The relationships between g_x, g_y and g_z in the $S = \frac{3}{2}$ scheme and g'_x, g'_y and g'_z in the $S' = \frac{1}{2}$ scheme are as follows:

$$g'_x = 2g_x(\beta^2 + 3\sqrt{3}\,\beta\alpha); \qquad g'_y = 2g_y(\beta^2 - \sqrt{3}\beta\alpha);$$

$$g'_z = g_z(\beta^2 - 3\alpha^2). \tag{4}$$

While the intrinsic g_x and g_y values are expected to deviate from the free electron value $g_e = 2.00$ owing to the spin-orbit coupling of the $^4A'_2$ ground state with the excited state, g_z is expected to be equal to g_e. Using first order perturbation theory:

$$g_\parallel = 2, \qquad g_\perp = 2(1 - 6\lambda'/\Delta_\perp), \tag{5}$$

$$D = \tfrac{1}{2}\lambda'[g_z - \tfrac{1}{2}(g_x + g_y)], \tag{6}$$

where λ' is the spin orbit coupling constant. This is smaller than the value for the free ion owing to covalent contributions to the bonding. $\Delta_\perp$ is the allowed optical transition $^4A'_2 \rightarrow {}^4E''$ which is x, y polarized.

To compare the stereochemistry of each of our systems, we need to evaluate for each the parameters γ and δ. The first, γ, gives the relative magnitude of the rhombic component; the second characterizes the system as tetra or pentacoordinate (i.e. approximately trigonal pyramidal or trigonal bipyramidal), as the value of δ is clearly larger in the pentacoordinated case.

The evaluation of these parameters requires the approximation $g_x = g_y$ in order to reduce the number of unknowns. This however does not introduce any significant error in calculating γ because the difference between g_x and g_y is mainly due to a different orbital contribution whilst that between g'_x and g'_y is due to the magnitude of mixing of the spin states; the same is true for δ, since the use of $2g_\perp$ instead of $g_x + g_y$ in eq. (6) is a good approximation.

4. Results and discussion

The ESR spectra at 7 K of Co(II) BCA at pH 6.0 and 9.5, and in the presence of 0.1 M NaI at pH 6.0 are shown in fig. 2. The spectrum of the low pH form shows axial symmetry with $g_\perp \approx 4$ and $g_\parallel \approx 2$. Therefore we have $\gamma = 0$ and $\beta^2 = 1$ (from eq. 3). In this case we cannot measure the δ parameter to assign the possible symmetry, but it is not incompatible with a

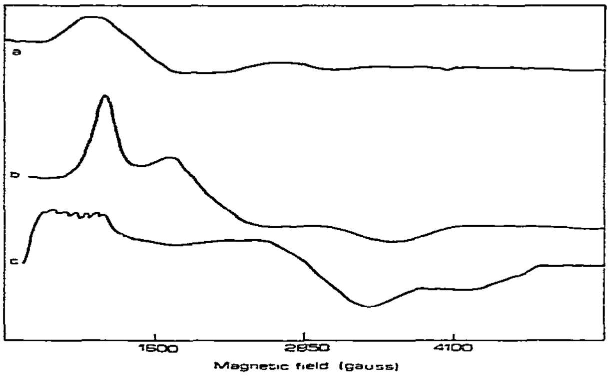

Fig. 2. ESR spectra of Co(II) BCA, 0.9 mM protein in water. a) as such (pH 6.0); b) brought to pH 9.5 by addition of concentrated NaOH; c) as (a) but in the presence of 0.1 M NaI. ESR conditions: frequency 9.24 Ghz, microwave power 6 mW, modulation amplitude 10 G. Temperature 7 K.

pseudotetrahedral geometry, as has been previously suggested [5,7]. On the other hand the spectra of the high pH form and of the iodide derivative show rhombic distortion. The spectrum of the iodide form presents also a clear hyperfine structure of about 92 gauss on the g_x component, due to the cobalt nuclear spin $I = 7/2$. Using the equations given above, we can now calculate the parameters γ and δ. From eq. (4) and the relation $\alpha^2 + \beta^2 = 1$, it is possible to calculate β^2, assuming the approximation $g_x = g_y$. We can then obtain γ from eq. (5), and δ from eq. (6) and the relation $\gamma = E/D$.

In eq. (6) we need to assign the right value to λ' which cannot be obtained from eq. (5) because the optical transition Δ_t is unknown. For the free Co(II) ion $\lambda' = -178$ cm^{-1}, but values for λ' are smaller than this owing to the orbital contributions to the bonding. The lowest likely value of λ' is about -135 cm^{-1} and the range of values calculated for δ were found by taking λ' between -179 cm^{-1} and -135 cm^{-1}. The experimental and calculated ESR data are reported in table 1 together with those of some tetra and pentacoordinate Co(II) compounds. The difference of δ values between the tetra and pentacoordinate complexes is immediately apparent, low values up to 17 cm^{-1} being associated with tetracoordinated species, whilst higher values of 50–81 cm^{-1} are associated with pentacoordination. Our values are larger than those usually found for tetracoordination [8,9] but not as large as for pentacoordination. This suggests that a fifth axial ligand in a trigonal bipyramidal structure is far removed and almost behaving as a trigonal pyramidal structure. A value of δ very similar to that of the iodide complex and of the high pH form is reported for

Table 1
Experimental[a] and calculated ESR parameters of high spin Co(II)

Compound[b]	g'_x	g'_y	g'_z	$g_\parallel$	$g_\perp$	β^2	γ	δ[c] (cm^{-1})	Ref.
CoBCA-I	6.82	2.26	1.56	2.25	2.46	0.923	0.362	44 – 33	this work
CoBCA-OH	5.60	3.20	1.85	2.04	2.25	0.976	0.190	39 – 30	this work
Pentacoordinate:									
Co(Et$_4$dien)Cl$_2$	7.08	2.53	1.59	2.19	2.58	0.931	0.357	81 – 62	[11]
Co(terpy)Cl$_2$	5.93	3.53	1.88	2.05	2.41	0.979	0.171	67 – 50	[11]
Tetracoordinate:									
CoCl$_2$(*m*-OMeA)$_2$	5.67	3.17	2.00	2.24	2.27	0.974	0.194	6 – 4	[9]
CoBr$_2$(*p*-MeA)$_2$	5.25	3.81	2.15	2.23	2.28	0.991	0.107	9 – 7	[9]
CoCl$_2$py$_2$	6.08	2.68	1.79	2.21	2.30	0.952	0.260	17 – 13	[8]
CoBr$_2$(2,4-lu)$_2$	6.79	1.65	1.29	2.30	2.37	0.890	0.450	16 – 12	[8]

[a] Determined graphically: g'_z on the high field minimum; g'_y on the central cross-over point; g'_x on the low field maximum for spectrum (b) and on the center of the hyperfine pattern for spectrum (c).

[b] Abbreviations used: Et$_4$dien = N,N,N′,N′-tetraethyldiethylenetriamine; terpy = terpyridyl; *m*-OMeA = *m*-methoxyaniline; *p*-MeA = *p*-methylaniline; py = pyridine; 2,4-lu = 2,4-lutidine.

[c] For $\lambda' = -179$ and -135 cm^{-1} respectively.

the acetazolamide complex of Co(II) BCA [10] and is considered to be indicative of pentacoordination. Another indication favouring pentacoordination is the presence of hyperfine structure in the iodide derivative which has never been detected in tetracoordinate compounds [11]. The divergence of the $g_{\parallel}$ calculated from eq. (4) from that of the free electron predicted by the first order perturbation theory is probably due to a lowering of the symmetry with a consequent increase [12] of the orbital contribution. In fact the difference is greater in the iodide derivative where the γ value, indicative of the degree of distortion, is greater than in the high pH form.

In conclusion, these data suggest that the Co(II) at the active site of BCA can be described as tetracoordinated with a fifth more distant ligand in agreement with the recent hypothesis [13] of a pentacoordinate intermediate in the enzymic reaction of carbonic anhydrase. This hypothesis is in agreement with the optical study [5] for the iodide derivative, but not for the high pH hydroxyl form. The discrepancy may be due to a different behaviour of the enzyme at low and high temperature as in the case of the CN^- derivatives [14], where there is a high spin-low spin conversion associated with the binding of one more CN^- group at low temperature.

Acknowledgement

This work has been partly supported by the NATO Research Grant No. 1379.

References

[1] J.E. Coleman, Inorganic biochemistry, vol. 1, ed. G.I. Eickhorn (Elsevier, London, 1973) p. 488.
[2] S. Lindoskog, Structure and Bonding (Berlin) 8 (1970) 153.
[3] S. Lindskog and B.G. Malmström, J. Biol. Chem. 237 (1962) 1129.
[4] A.E. Dennard and R.J.P. Williams, Transition Metal Chem. 2 (1966) 115.
[5] I. Bertini, C. Luchinat and A. Scozzafava, Inorg. Chim. Acta 22 (1977) 223.
[6] L. Morpurgo, G. Rotilio, A. Finazzi Agrò and B. Mondovi, Arch. Biochem. Biophys. 170 (1975) 360.
[7] B. Holmquist, T.A. Kaden and B.L. Vallee, Biochemistry 14 (1975) 1454.
[8] Yu.V. Yablokov, V.K. Voronkova, V.F. Shiskhov, A.V. Ablov and Zh.Yu. Vaisbein, Sov. Phys. Solid State 13 (1971) 831.
[9] V.K. Voronkova, M.M. Zaripov, Yu.V. Yablokov, A.V. Ablov and M.A. Ablova, Dokl. Akad. Nauk. SSSR 214 (1974) 377.
[10] R. Aasa, M. Hanson and S. Lindskog, Biochim. Biophys. Acta 453 (1976) 211.
[11] F.S. Kennedy, H.A.O. Hill, T.A. Kaden and B.L. Vallee, Biochem. Biophys. Res. Comm. 48 (1972) 1533.
[12] Z. Dory and H.B. Gray, Inorg. Chem. 7 (1968) 889.
[13] K.K. Kannan, M. Petef, K. Fridborg, H. Cid-Dresdner and S. Lövgren, FEBS Letters 73 (1977) 115.
[14] P.H. Haffner and J.E. Coleman, J. Biol. Chem. 250 (1975) 996.