STEREOCHEMISTRY OF ANION COMPLEXES OF TYPE 2 Cu(II) IN *RHUS VERNICIFERA* LACCASE

Analogy with superoxide dismutase and Cu(II) carbonic anhydrase

A. DESIDERI, L. MORPURGO, G. ROTILIO and B. MONDOVÌ

Department of Physics, University of Calabria, Arcavacata, Center of Molecular Biology of the CNR, Rome and *Institutes of Biological Chemistry and of Applied Biochemistry, University of Rome, Rome, Italy*

Received 19 December 1978

1. Introduction

Rhus vernicifera laccase is a multicopper oxidase containing three types of copper [1]; Type 1 Cu(II), unaccessible to solvent molecules and with unusual spectroscopic parameters such as a very high extinction coefficient at 614 nm and a low parallel hyperfine constant ($A_{\parallel}$) of the EPR spectrum; type 2 Cu(II) with 'usual' EPR and optical parameters, open to solvent access; type 3 Cu(II), undetectable by EPR and probably consisting of a pair of magnetically-coupled cupric ions [2]. While the blue copper has generated a great deal of interest because of its unique properties and is now precisely classified as far as structure and functions are concerned [1], the type 2 Cu(II) is rather defined by the absence of peculiar properties. In the search for a positive approach to its characterization, we have reexamined the reactions with anions of laccase, as such reactions have proved useful in the study of the metal site of many enzymes, when it was capable to bind solvent molecules. It was found that type 2 Cu(II) forms with N_3^- and CN^-, under reducing conditions peculiar complexes for which a pentacoordinate geometry is proposed by analogy of EPR properties to the copper binding sites of superoxide dismutase and carbonic anhydrase in situations of very likely pentacoordination.

2. Materials and methods

Rhus vernicifera laccase, supplied as the acetone powder of the lecquer by Saito and Co., Japan, was purified according to [3]. Anaerobic experiments were carried out in an EPR tube sealed to a Thunberg type apparatus, that was deareated by three vacuum-flushing cycles with purified argon. Deareated ferrocyanide solutions were added with an air-tight microsyringe through serological caps. X-band EPR spectra were recorded on a Varian E-9 spectrometer, equipped with the Varian variable temperature accessory.

3. Results

Figure 1 shows the EPR spectra of 0.2 mM laccase in 0.05 M phosphate pH 7.5 (curve a) and of the enzyme frozen immediately after the addition of a 10-fold excess NaCN, where the signal due to type 2 Cu is apparently modified (curve b). Incubation with a larger NaCN excess resulted in a slow reduction of both type 1 and type 2 Cu signals, paralleled by a decrease in A_{614}. The degree of reversibility of the reaction decreased with increasing [NaCN] and incubation time.

When 4.0 mM NaCN was added to the enzyme anaerobically reduced with excess ferrocyanide a new derivative was formed. Its EPR spectrum (curve c) is characterized by a low $A_{\parallel}$ value of 139×10^{-4} cm^{-1} (table 1) and accounts for 50% of the original EPR-detectable Cu. Overnight dialysis against 0.1 M phosphate (pH 6.0) restored about 78% of the visible absorption and 90% of EPR detectable copper with good reversibility of the initial line shape (curve d).

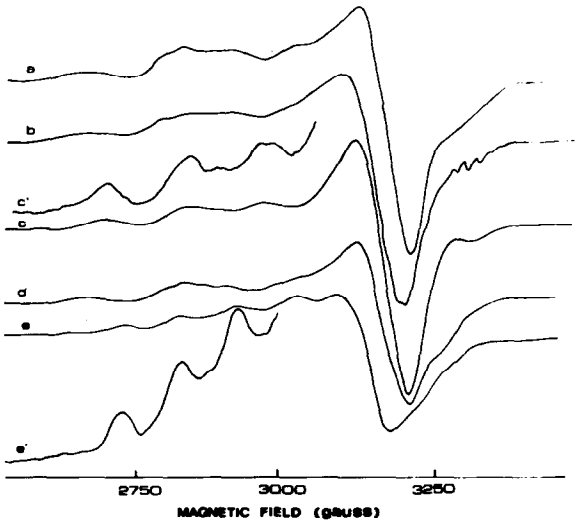

Fig.1. EPR spectra of laccase and of its CN^- and N_3^- derivatives. (a) 0.2 mM laccase in 0.05 M phosphate buffer (pH 7.5); (b) 2.0 mM NaCN added anaerobically; (c) anaerobically reduced with 4.0 mM ferrocyanide, then 4.0 mM NaCN added, amplification factor × 2; (c') × 5; (d) after overnight dialysis against 0.1 M phosphate buffer (pH 6.0); (e) 0.2 mM laccase in 0.05 M acetate buffer (pH 4.5) and 2.0 mM NaN_3 anaerobically reduced with 2.0 mM ferrocyanide; (e') = (e) × 5. The intensity of spectra (d) and (e) is not directly comparable to that of the other spectra because of different experimental conditions. The intensity ratios reported in the text were calculated with reference to Cu–EDTA standards. Setting conditions: microwave frequency 9.15 GHz; microwave power 20 mW; temperature 100 K.

Table 1
EPR parameters in the low field region of some Cu(II) enzymes

Protein	g_z	$\lvert A_z \rvert$ ($cm^{-1} \times 10^{-4}$)	Ref.
Laccase			
Type 2 Cu(II)	2.237	200	[1]
Type 2 Cu(II)·N_3	2.276	106	This work
Type 2 Cu(II)·CN	2.268	139	This work
Carbonic anhydrase			
Cu(II)·N_3	2.284	106	[11]
Cu(II)·CN	2.234	127	[11]
Superoxide dismutase			
2 Zn(II) 2 Cu(II)	2.265	137	[10]
2 Cu(II) 2 Cu(I)	2.31	108	[13]

The total content of copper of the treated sample was 82% of the initial value after correction for dilution. When larger amounts of NaCN and ferrocyanide were used the extent of reduction was proportionally higher and the degree of reversibility lower.

Curve a shows the spectrum of laccase anaerobically treated with ferrocyanide in the presence of NaN_3 at pH 4.5. As reported in [4] the reaction only caused a 35% loss of total EPR signal intensity, probably due to residual type 1 Cu(II). The spectrum is characterized by a low $A_\parallel$ of 106×10^{-4} cm^{-1} (table 1) and by a distinct rhombic line shape.

Attempts to prepare the derivatives with other anions such as CNS^- or I^- were unsuccessful. When the protein reduced with ferrocyanide in the presence of these anions was frozen, a change of colour from yellow to green was observed. The EPR signal showed in any case a decrease of intensity of only 10–15% and the presence of at least two species. One species was certainly type 1 Cu(II), the other one probably a modified form of type 2 Cu(II). The EPR parameters of the latter species could not be determined due to very poor signal resolution.

4. Discussion

The reactivity of tree laccase with cyanide is quite different from that of fungal laccase [5]. In the latter enzyme high concentrations of cyanide caused a complete and reversible reduction of only the type 1 Cu, whilst type 2 Cu, bound to two CN^- [6], resisted reduction. In the presence of ferrocyanide, however, a similar reaction was also obtained with tree laccase. The new EPR signal due to type 2 Cu(II) is considerably different and like that obtained in the presence of NaN_3 is characterized by a low $A_\parallel$ value, such as is only rarely found in low molecular weight copper complexes. It seems to be somewhat more frequent among copper-containing enzymes and their derivatives. In table 1 the EPR parameters at low field of such enzymes are listed.

For the Cu(II) carbonic anhydrase derivatives of table 1 a pentacoordinate structure was suggested on the basis of NMR measurements [8]. A pentacoordinate structure was also suggested for native superoxide dismutase by X-ray diffraction studies [9]. The rhombicity of the EPR spectra of the above compounds

[10,11] suggests that their symmetry is quite low. In C_{3v} geometry it is possible to account for the low value of $A_{\parallel}$ and for the rhombicity of the spectra observed, as the $d_{x^2-y^2}$ orbital can mix with the d_{z^2} orbital. In fact with a ground state function of the form:

$$\psi = \alpha\,|d_{x^2-y^2}> + \beta\,|d_{z^2}>$$

the following equations are obtained for the spin Hamiltonian parameters:

$$g_x = 2.0023 - 2\,\lambda(\sqrt{3}\beta + \alpha)^2/\Delta E_{yz}$$

$$g_y = 2.0023 - 2\,\lambda(\sqrt{3}\beta - \alpha)^2/\Delta E_{xz}$$

$$g_z = 2.0023 - 8\,\lambda\alpha^2/\Delta E_{xy}$$

$$A_z = [P - K - (\alpha^2 - \beta^2)\,4/7]$$

Compared to a pure $d_{x^2-y^2}$ ground state, the absolute value of the parallel hyperfine constant A_z is smaller, as $|\alpha^2 - \beta^2|$ is certainly <1 and tends to disappear depending on the extent of mixing between the $d_{x^2-y^2}$ and d_{z^2} orbitals. At the same time an increase of rhombicity occurs, consistent with the spectra of the compounds examined.

For the iodide derivative of Cu(II) carbonic anhydrase a pseudo-trigonal bipyramidal geometry was suggested with a d_{z^2} ground state [12]. This configuration is not possible for Cu(II) carbonic anhydrase derivatives reported in table 1 since the lowest g-value is larger [11] than the value of the free electron.

The parameters reported in table 1 are also consistent with a pseudo-tetrahedral geometry [7] where the 4p and 3d orbitals can mix as they have the same symmetry. This is probably the case of Cu(II) substituted in the tetrahedral Zn(II) site [9] of partially-reduced superoxide dismutase [13], indicated in table 1 as the 2 Cu(II) 2 Cu(I) enzyme.

Turning to the two anionic derivatives of laccase type 2 Cu(II), it is difficult to state whether their actual geometry is pseudo-tetrahedral or penta-coordinate. The close similarity of A_z with the corresponding derivatives of Cu(II) carbonic anhydrase would favour pentacoordinate geometry. In any case the high affinity for anions of the partially reduced protein, similar to that observed with carbonic anhydrase, is indicative of a positively charged metal site in a hydrophobic environment [4,14].

Acknowledgement

This investigation was supported in part by the 'Contributo per la ricerca scientifica, Cap. 9 art. 15 Bilancio Universitario'. The technical assistance of Mr V. Peresempio is gratefully acknowledged.

References

[1] Fee, J. A. (1975) Struct. Bond. 23, 1–60.
[2] Solomon, E. I., Dooley, D. M., Wang, R. H., Gray, H. B., Cerdonio, M., Mogno, F. and Romani, G. L. (1976) J. Am. Chem. Soc. 98, 1029–1031.
[3] Reinhammar, B. (1970) Biochim. Biophys. Acta 205, 35–47.
[4] Morpurgo, L., Rotilio, G., Finazzi Agrò, A. and Mondoví, B. (1974) Biochim. Biophys. Acta 336, 324–328.
[5] Malmström, B. G., Reinhammar, B. and Vänngård, T. (1968) Biochim. Biophys. Acta 136, 67–76.
[6] Malkin, R., Malmström, B. G. and Vänngård, T. (1968) FEBS Lett. 1, 50–54.
[7] Attanasio, D., Tomlinson, A. A. G. and Alagna, L. (1977) Chem. Commun. 618–619.
[8] Bertini, I., Canti, G., Lucchinat, C. and Scozzafava, A. (1979) J. C. S. Dalton, in press.
[9] Richardson, J. S., Thomas, K. A., Reubin, B. H. and Richardson, D. C. (1975) Proc. Natl. Acad. Sci. USA 72, 1349–1353.
[10] Rotilio, G., Morpurgo, L., Giovagnoli, C., Calabrese, L. and Mondoví, B. (1972) Biochemistry 11, 2187–2192.
[11] Haffner, P. H. and Coleman, J. E. (1975) J. Biol. Chem. 250, 996–1005.
[12] Murpurgo, L., Desideri, A., Falcioni, R., Rotilio, G. and Mondoví, B. (1978) Inorg. Chim. Acta 28, L141–L143.
[13] Fee, J. A. and Briggs, R. G. (1975) Biochim. Biophys. Acta 400, 439–450.
[14] Kannan, K. K., Petef, M., Fridborg, K., Cid-Dresdner, H. and Löwgren, S. (1977) FEBS Lett. 73, 115–119.