THE STOICHIOMETRY OF THE THREE DIFFERENT TYPES OF COPPER IN ASCORBATE OXIDASE FROM GREEN ZUCCHINI SQUASH

J. DEINUM and B. REINHAMMAR

Institutionen för Biokemi, Göteborgs universitet och Chalmers Tekniska Högskola, Fack, S-402 20, Göteborg 5, Sweden

and

A. MARCHESINI

Instituto Sperimentale per la Valorizzazione Tecnologica dei Prodotti Agricoli, Via Celoria 2 201 33 Milano, Italy

Received 19 April 1974

1. Introduction

Ascorbate oxidase (L-ascorbate: O_2 oxidoreductase EC 1.10.3.3) from green zucchini squash, *Cucurbita pepo medullosa,* belongs to the group of enzymes usually referred to as the 'blue' copper-containing oxidases. The other members of this group are the laccases and ceruloplasmin [1], which have been studied extensively. These latter enzymes possess very similar optical spectral properties related to the copper moiety. Another common feature is the presence of two different types of EPR detectable copper: Type 1 or 'blue' copper, showing unusual narrow hyperfine structure and Type 2 'non blue', which does not seem to contribute very much to the optical absorbance, and has a more 'normal' EPR spectrum. The EPR non-detectable copper is thought to be associated with the absorbance band at 330 nm [2]. At least part of the copper is involved in the transfer of electrons from the substrate to oxygen, the final electron acceptor [3].

Less is known about the state of copper in ascorbate oxidase. The enzyme has a mol. wt. of 140 000 and a copper content of 8–10 atoms per molecule [4]. Published EPR spectra of ascorbate oxidase [5–7] show the presence of both Type 1 and Type 2 Cu^{2+}. However, no investigation has been made of the stoichiometry of the different types of copper, and no attempt was made to simulate the EPR spectrum. Therefore we considered it of particular interest to investigate this aspect, as it probably will help to elucidate the role of the different types of copper in the reaction mechanism of all 'blue' copper-containing oxidases.

2. Materials and methods

2.1. *Electron paramagnetic resonance (EPR) measurements and simulations*

EPR measurements were made at about 9 GHz at 77 and 10°K in a Varian E-3 spectrometer and at about 35 GHz at 90°K in a Varian V-4503 spectrometer.

Computer simulations were performed with a HP 9100 B–9125 A desk calculator and an IBM 360/65 computer using a program developed by Tore Vänngård [8].

2.2. *Other spectral measurements*

All measurements were performed at room temperature. Visible and ultraviolet spectra were obtained with a Zeiss RPQ 20 A recording spectrophotometer. Fluorescence emission and excitation spectra were obtained with an Aminco Bowman spectrofluorimeter equipped with a Xenon lamp. No correction was applied for lamp fluctuations.

2.3. *Protein*

Three preparations of ascorbate oxidase from green zucchini squash, *C. pepo medullosa,* were made by the method of Marchesini [9]. The protein was further chromatographed on DEAE-Sephadex A 50 (Pharmacia, Sweden) in 0.05 M phosphate buffer at pH 7.0 and a 0–0.2 M NaCl gradient which removed traces of contaminating chromophores. The ratio of the absorption at 280 and 610 nm was 25 ± 0.5. Specific activity measurements were performed according to Dawson and Magee [10] and the obtained value, 900 units per μg copper, was in agreement with values published by Lee and Dawson [4].

2.4. *Copper determinations*

Total copper was determined with 2,2′-biquinoline [11]. EPR detectable copper was determined by double integration of the 9 GHz EPR spectrum. A 1 mM copper solution in 0.01 M HCl and 2 M $NaClO_4$ was used as a standard, and corrections for the different g factors [1] were applied.

2.5. *Chemicals*

All chemicals were of analytical grade and were used without further purification. Solutions were made with deionised, distilled water.

3. Results and discussion

From the optical absorption spectrum (fig. 1) and the copper determinations, the extinction coefficients per total copper for the oxidized minus reduced ascorbate oxidase were calculated: 1.21 $mM^{-1} \cdot cm^{-1}$

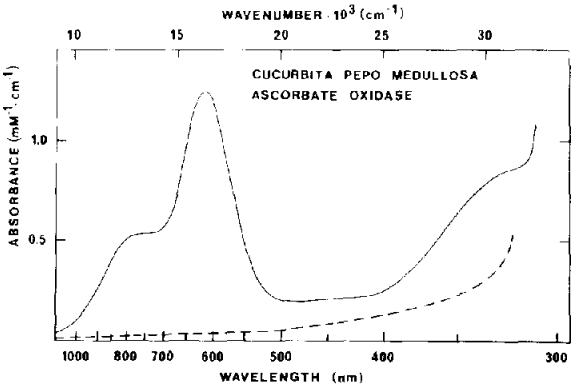

Fig. 1. Optical absorption spectrum of ascorbate oxidase from green zucchini squash in 0.1 M phosphate buffer pH 7.0. The absorbance is given per mM of total copper. (— —) Oxidized protein. (– – – –) Reduced protein, after addition of small amounts of solid ascorbic acid. The spectrum is the same for the protein in 0.1 M Tris–glycine buffer pH 8.6.

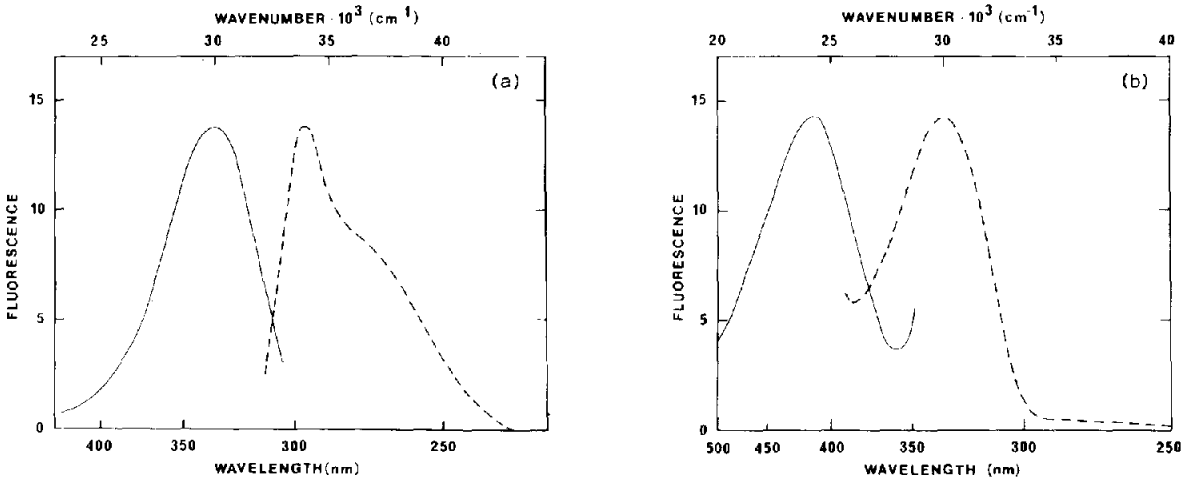

Fig. 2. Fluorescence spectra of ascorbate oxidase from green zucchini squash in 0.1 M phosphate buffer pH 7.0: (a) – – – –, excitation spectrum with λ_{em}^{max} = 335 nm. ——, emission spectrum with λ_{ex}^{max} = 295 nm. The absorbance at 280 nm was 0.5 cm^{-1}; (b) – – – –, excitation spectrum with λ_{em}^{max} = 415 nm. ——, emission spectrum with λ_{ex}^{max} = 335 nm. The absorbance at 335 nm was 0.6 cm^{-1}. The fluorescence is given in arbitrary units.

at 610 nm and 0.49 $mM^{-1} \cdot cm^{-1}$ at 330 nm. There is no indication of an absorption band centred at 880 nm as found by Lee and Dawson [5]. Lee and Dawson's [4,5] preparation seems to be less pure than our preparation as it has also a much higher absorption in the visible spectrum after reduction of the copper moiety with ascorbic acid. Another indication that our preparation is different comes from fluorescence measurements. As is shown in fig. 2a the aromatic amino acids excited at the maximum at 295 nm gave an emission spectrum with a maximum at 335 nm rather than 325 nm [5]. An interesting finding is that ascorbate oxidase, as for example the laccases, ceruloplasmin and stellacyanin (unpublished, J. Deinum and B. Reinhammar), shows an excitation maximum at about 335 nm with an emission maximum at about 415 nm (fig. 2b). This fluorescence is still present in the reduced protein or after denaturation in 6 M guanidine HCl. Thus the chromophore is associated with the protein part of the enzyme rather than with the copper. The absorption at the wavelength region 310 nm and upwards, which cannot be associated with the normal tryptophan or tyrosin residues [12], has received very little attention. This absorption might be due to the presence of modified aromatic amino acid residues or other unknown components.

Double integrations of 9 EPR spectra recorded at about 9 GHz revealed that 47 ± 3% of the total copper was paramagnetic. The first hyperfine line of Type 2 Cu^{2+} at low-field is well separated from the rest of the spectrum (fig. 3a and a'), and integrations [13] of this line showed that 25 ± 5% of the total EPR spectrum originates from Type 2 Cu^{2+}. The EPR spectra at 9 and 35 GHz can be simulated assuming three times as much Type 1 Cu^{2+} as Type 2 Cu^{2+} (fig. 3b and b' and fig. 4b and b'). Table 1 shows the g and A values which were used in the simulations.

Our data are consistent with the presence of 8 copper atoms per molecule of ascorbate oxidase. Four of these are EPR nondetectable, three have Type 1 Cu^{2+} and one has Type 2 Cu^{2+} character [1]. The three Type 1 Cu^{2+} in ascorbate oxidase appear to have identical EPR parameters, which does not mean, of course, that they must be identical in other aspects. The two Type 1 in ceruloplasmin, for example, are different both with respect to their g and A values and their redox behaviour, although they have

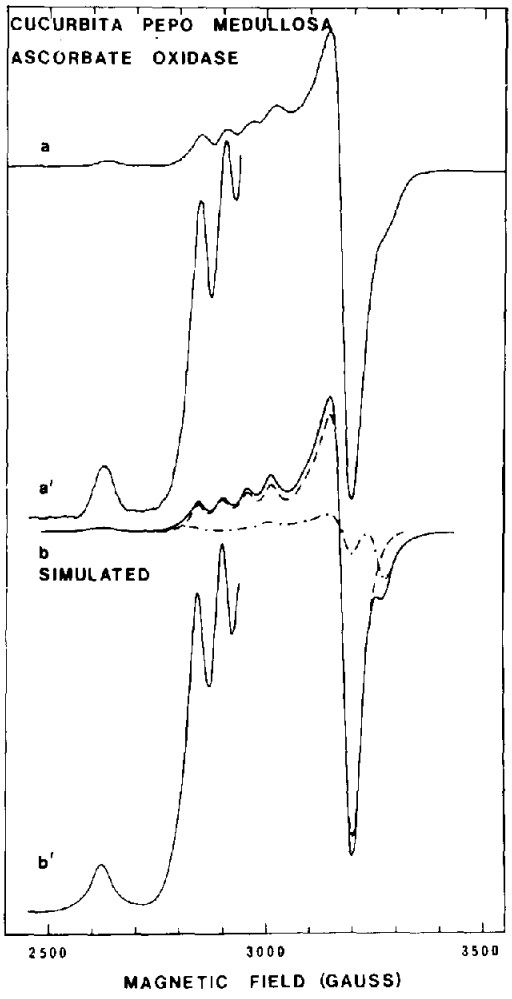

Fig. 3. Experimental (a) and simulated (b) EPR spectrum of ascorbate oxidase from green zucchini squash at about 9 GHz and 77°K. The experimental spectrum (a) was obtained with ascorbate oxidase in 0.1 M Tris–glycine buffer pH 8.6 and is the same for the protein in 0.1 M phosphate buffer, pH 7.0. The simulated spectrum (b) is the sum of two components, Type 1 (———) and Type 2 Cu^{2+} (—·—) with relative intensities of 3 and 1 resp. Lorentzian line shape was assumed. The parameters used in the simulations are given in table 1. The linewidth for Type 1 and Type 2 Cu^{2+} was 25 and 35 Gauss resp. Part of the spectrum is also shown with 10 times higher gain, (a') and (b'). Microwave frequency was 9.122 GHz, microwave power 10 mW, and modulation amplitude 10 Gauss. The sample contained 0.95 mM EPR-detectable copper which was 48% of the total copper content.

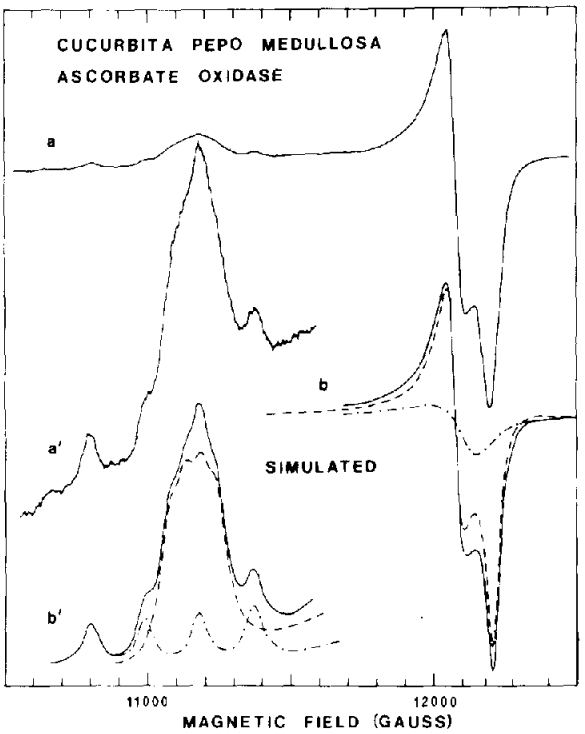

Fig. 4. Experimental (a) and simulated (b) EPR spectrum of ascorbate oxidase from green zucchini squash at about 35 GHz and 90° K. Spectrum (b) was simulated as in fig. 3 except that Type 1 and Type 2 Cu^{2+} were given linewidths of 50 and 40 Gauss resp. in the $g_{//}$ region and 30 and 100 Gauss in the $g_{\perp}$ region. Part of the spectrum is shown with higher gain, (a') and (b'). Microwave frequency was 34.778 GHz, microwave power 10 mW and modulation amplitude 2.5 Gauss. The sample was the same as used in fig. 3.

the same extinction coefficient at 610 nm [14].

The EPR spectrum reported here is very similar to that of ascorbate oxidase from *Cucumis sativus*, as published by Nakamura [6]. Most likely, the proteins from the two sources have the same ratio of Type 1 to Type 2 Cu^{2+}. On the other hand, the EPR spectra published by Lee and Dawson [5] and Avigliani et al. [7] show one more low-field line and a much broader signal in g_1 region. However, a spectrum of this character was also found in our protein after storage for a couple of weeks at 4°C in 0.2 M NaCl + 0.01 M phosphate buffer, pH 7.0, which irreversibly changed the EPR spectrum. Assuming that all three Type 1 Cu^{2+} contribute equally to the absorption at 610 nm the extinction coefficient per Type 1 Cu^{2+} is 3.3 $mM^{-1} \cdot cm^{-1}$. This value lies in the range of the blue proteins containing only Type 1 Cu^{2+} [1], but is considerably lower than the values found for the laccases and ceruloplasmin [1].

Table 1
The EPR parameters used for simulation of the EPR spectra of ascorbate oxidase from green zucchini squash, *Cucurbita pepo medullosa*

Type 1 Cu^{2+}			Type 2 Cu^{2+}	
g_x 2.036	g_y 2.058	g_z 2.227	$g_{\perp}$ 2.053	$g_{//}$ 2.242
A_x 5	A_y 5	A_z 56	$A_{\perp}$ 10	$A_{//}$ 190

The absolute values of the hyperfine constants are given in Gauss.

The presence of only one Type 2 Cu^{2+} in ascorbate oxidase is inconsistent with a symmetrical quaternary structure, consisting of two identical units, two 'laccase' halves, as proposed by Strothkamp and Dawson [15]. In fact all the blue oxidases seem to have only one Type 2 Cu^{2+} per molecule. On the other hand, the number of Type 1 Cu^{2+} differs among the blue oxidases. Thus the laccases contain one, ceruloplasmin two and ascorbate oxidase three Type 1 Cu^{2+} per molecule. Several inhibitors like F^-, N_3^- and CN^- are shown to interact with Type 2 Cu^{2+} [16–18]. Binding of one F^- to Type 2 Cu^{2+} in fungal laccase [17] completely inhibits the enzyme. Therefore this copper ion must have an important role in the catalytic mechanism.

It has earlier been proposed that Type 2 Cu^{2+} in fungal laccase might play a role in stabilisation of an oxygen intermediate formed during reduction [19]. However, this has not been proven unambiguously and needs to be further investigated also in the other blue oxidases.

Acknowledgements

This study has been supported by grants from the Swedish Natural Science Research Council. A short time EMBO fellowship to one of us (A.M.) is gratefully acknowledged. We are indebted to Dr T. Vänngård for his criticism and valuable discussions.

References

[1] Vänngård, T. (1972) in: Biological Applications of Electron Spin Resonance (Swartz, H. M., Bolton, J. R. and Borg, D. C., eds), pp. 411–447, John Wiley & Sons, New York.

[2] Malkin, R., Malström, B. G. and Vänngård, T. (1969) Eur. J. Biochem. 10, 324–329.

[3] Andréasson, L.-E., Malmström, B. G., Strömberg, C. and Vänngård, T. (1973) Eur. J. Biochem. 34, 434–439.

[4] Lee, M. H. and Dawson, C. R. (1973) J. Biol. Chem. 248, 6596–6602.

[5] Lee, M. H. and Dawson, C. R. (1973) J. Biol. Chem. 248, 6603–6609.

[6] Nakamura, T., Makino, N. and Ogura, Y. (1968) J. Biol. Chem. 64, 189–195.

[7] Avigliani, L., Gerosa, P., Rotilio, G., Finazzi Agrò, A., Calabrese, L. and Mondovi, B. (1972) Ital. J. Biochem. 21, 248–255.

[8] Vänngård, T. and Aasa, R. (1963) in: Paramagnetic Resonance (Low, W. ed.), Vol. 2, pp. 509–519, Academic Press, New York.

[9] Marchesini, A. (1974) submitted to Biochim. Biophys. Acta.

[10] Dawson, C. R. and Magee, R. J. (1955) Methods Enzymol. 2, 831–835.

[11] Brumby, P. E. and Massey, V. (1967) Methods Enzymol. 10, 473–474.

[12] Handbook of Biochemistry (1970) (Sober, H. A. ed.) p. B 75, The Chemical Rubber Company, Cleveland, Ohio.

[13] Vänngård, T. (1967) in: Magnetic Resonance in Biological Systems, (Ehrenberg, A., Malström, B. G. and Vänngård, T., eds.), pp. 213–215, Pergamon, Oxford.

[14] Deinum, J. and Vänngård, T. (1973) Biochim. Biophys. Acta 310, 321–330.

[15] Strothkamp, K. G. and Dawson, C. R. (1974) Biochemistry 13, 434–440.

[16] Andréasson, L.-E. and Vänngård, T. (1970) Biochim. Biophys. Acta 200, 247–257.

[17] Brändén, R., Malmström, B. G. and Vänngård, T. (1973) Eur. J. Biochem. 36, 195–200.

[18] Malmström, B. G., Reinhammar, B. and Vänngård, T (1968) Biochim. Biophys. Acta 156, 67–76.

[19] Andréasson, L.-E., Brändén, R., Malmström, B. G. and Vänngård, T (1973) FEBS Letters 32, 187–189.