REMOVAL OF NON-BLUE COPPER FROM ASCORBATE OXIDASE

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1. Introduction

Ascorbate oxidase (AAO, EC 1.10.3.3) contains at least 3 different types of copper classified as type 1, type 2 and type 3 according to the Malmström proposal [1]; the stoichiometry of this copper is not yet clear. In order to get new information on this problem, the possibility of a selective removal of the metal with a method described for *Rhus vernicifera* laccase [2] was investigated. The metal-depleted ascorbate oxidase appears to have lost type 2 copper.

2. Materials and methods

Reagent grade chemicals were used without further purification. Ascorbate oxidase was purified from green zucchini by the method of A. M. and Kroneck (to be published).

Protein concentration was determined assuming $E_{280} = 24 \times 10^4$ cm^2 M^{-1}, $E_{605} = 10^4$ cm^2 M^{-1} [3] and by biuret method [4]. Only enzyme samples with an $A_{280}/A_{605} \leqslant 28$ and homogeneous on disc gel electrophoresis at pH 8.9 were used. A mol. wt 140 000 was assumed [3].

Optical spectra were recorded with a Cary 14 spectrometer and X band EPR spectra with V 4502 Varian Spectrometer.

The ascorbate oxidase activity was followed by recording the decrease at A_{295} related to the ascorbate oxidation.

Copper content was determined by 2,2′-bichinolin reaction [5]. Native protein was dialyzed against 500 μM EDTA in order to remove heterogeneous copper present.

The copper-depleted AAO was prepared by 12 h anaerobic dialysis of 100 μM protein sample against a solution containing 2 mM dimethylglyoxine (DMG), 1 mM EDTA and 5 mM ferrocyanide in 50 mM acetate buffer (pH 5.2).

The protein solution was then aerobically dialyzed against 0.1 M phosphate buffer (pH 6). Reconstituted enzyme was obtained by anaerobic addition of 10 equiv. $CuCl_2$ in the presence of ascorbate and by dialysis against 500 μM EDTA to remove the unspecifically bound copper.

3. Results and discussion

The EPR spectrum of an EDTA–DMG treated sample of ascorbate oxidase is reported in fig.1. By comparison with that of an untreated sample it is evident that the absorption related to type 2 copper is absent. On the other hand the absorption spectrum shows a decrease of the 330 nm band, which has been ascribed to the type 3 copper [6]. A decrease at 750 nm is also observed. Chemical and EPR determinations of the copper content in treated and untreated protein samples reported in table 1, indicate a loss of copper atoms. Some samples however show a lower total copper content after EDTA–DMG treatment. It appears therefore that the treatment removes almost selectively the type 2 copper from ascorbate oxidase as reported for laccase [2], though some type 3 copper could be also removed. Since the

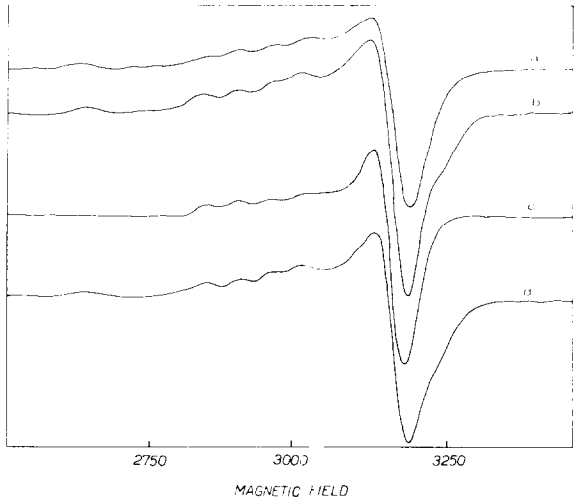

Fig.1. X band EPR spectra of ascorbate oxidase. (a) Native ascorbate oxidase in 0.1 M acetate buffer (pH 5.2). (b) After treatment with EDTA. (c) After treatment with EDTA–DMG. (d) After recombination. Temperature, 77 K. Modulation amplitude, 10 G. Microwave power, 20 mW. The intensities of the spectra are not directly comparable because of different experimental conditions. The intensity ratios, reported in table 1, were calculated with reference to Cu–EDTA standards.

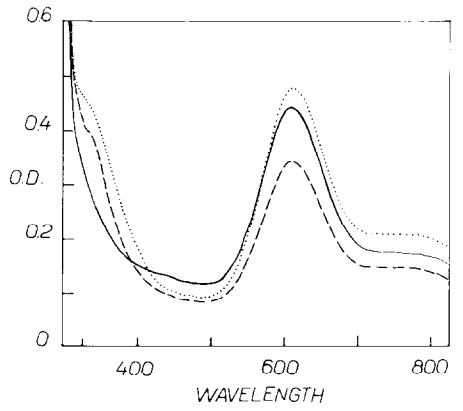

Fig.2. Absorbtion spectra of ascorbate oxidase. (· · ·) Native ascorbate oxidase in 0.1 M acetate buffer pH 5.2. (——) After treatment with EDTA–DMG. (– – –) After recombination. Molar concentrations of the samples are reported in table 1. Optical path., 1 cm.

absorbance decrease is not related to the amount of copper lost, it appears that type 2 copper contributes to the 330 and 750 nm absorption bands.* At present it is impossible to state what is the relationship between the two. It is important to recall that the blue band and the A_{280}/A_{605} ratio are unaffected by this treatment, which causes a 95% decrease of ascorbate oxidase activity. Treatment with EDTA alone reduces the total copper content to 6–7 copper atoms/mol enzyme affecting neither the absorbance spectrum nor the oxidase activity, while the EPR spectrum is somewhat modified (fig.2). Double integration of the spectrum shows a decrease of EPR detectable copper which could be considered as spurious since after its removal the type 2 signal appears 'clearer'.

It appears that the reports of 8–12 copper atoms/enzyme molecule do not take into account this possibility [7].

Readdition of copper to the copper-depleted ascorbate oxidase restores the original EPR and optical spectra and the activity. As reported in table 1 the

* Similar results have been obtained with *Rhus vernicifera* laccase by Morpurgo et al. (to be published)

Table 1
Properties of native and copper-depleted ascorbate oxidase

Protein	$\frac{A_{280}}{A_{330}}$	$\frac{A_{280}}{A_{605}}$	Protein (μM)[a]	Copper content (μM)	
				Total[b]	EPR detectable[c]
Native	29.5	27	48	370	247
EDTA-treated	29.5	27	48	326	205
EDTA–DMG-treated	40	26.8	44	198	110
Recombined	28	29	34	238	129

[a] By biuret method (similar results were obtained by $E^{M}_{280\ nm}$)
[b] By bichinolin method
[c] By EPR double integrated intensity

copper content of the EDTA treated and the reconstituted samples amounts to 6–7 copper/mol protein. Double integration of their EPR spectra indicates that 4.5 coppers are EPR detectable. The EDTA–DMG-treated samples show instead a total copper content of about 5 ions with about 3 paramagnetic (type 1) atoms.

Though some variability was observed among different samples, ascorbate oxidase seems to contain three type 1 Cu^{2+}, one type 2 and a pair of magnetically-coupled copper atoms. A ratio of 3:1 for type 1/type 2 copper has been suggested [8,9].

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