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Single crystal absorption spectra of ascorbate oxidase from green zucchini squash

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Single crystal polarized absorption spectra of the dimeric multicopper enzyme ascorbate oxidase from green zucchini squash indicate that its most relevant functional and structural properties are maintained in the crystalline state. Since the polarized absorption spectra of crystalline ascorbate oxidase are very similar, in the visible region, to those of crystalline plastocyanin, we expect that structural data will show similar orientation of the type 1 Cu^{2+} center with respect to the crystal axes. The selective removal of type 2 Cu^{2+} from the crystal has been realized and has a potential value for the identification of the copper centers in the crystallographic analysis of the enzyme. Evidence is presented for an azide binding site formed by type 2 and type 3 Cu^{2+}, similar to the trinuclear copper center suggested to be present in laccase.

Ascorbate oxidase; Multicopper enzyme; Enzyme crystal; Microspectrophotometry

1. INTRODUCTION

Ascorbate oxidase is a copper containing enzyme belonging to the class of blue oxidases as the other animal and plant enzymes, ceruloplasmin and laccase [1]. The Cu^{2+} are of three different types with distinct spectroscopic features [2]. Type 1 Cu^{2+} shows high absorption in the visible region ($\epsilon > 3000\ M^{-1} \cdot cm^{-1}$ at 600 nm) and an EPR spectrum with narrow hyperfine lines ($A_{\parallel} < 90$ G); type 2 Cu^{2+} has undetectable optical absorption and an EPR line shape of the usual low molecular mass copper complexes ($A_{\parallel} > 140$ G); type 3 Cu^{2+} is characterized by a strong absorption in the near UV region ($\lambda_{max} = 330$ nm) and by the absence of an EPR signal, due to the antiferromagnetic coupling of two Cu^{2+}. All the above mentioned features disappear upon reduction of copper with suitable electron donors [2].

Laccase is the simplest component of this protein family, containing 4 Cu^{2+} with the following stoichiometry: one type 1, one type 2 and two type 3 Cu^{2+} [1]. Though similar, the situation with the other blue oxidases is complicated by the presence of a higher content of Cu^{2+}.

The dimeric ascorbate oxidase (140 kDa) was reported to contain 8 Cu^{2+} per dimeric unit [3]. The simplest interpretation of this fact would be that each monomer is a laccase-like unit. Some evidences, however, contradict this view [4,5].

In the wait for structural information to be extracted from X-ray crystallographic studies in progress, we have investigated the optical absorption properties of ascorbate oxidase by single crystal microspectrophotometry in polarized light in order to compare some properties of the enzyme in the crystal and in solution.

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2. MATERIALS AND METHODS

Ascorbate oxidase from *Cucurbita pepo medullosa* was prepared according to Avigliano et al. [6] and crystallized in 50 mM phosphate buffer, pH 5.8, by dialysis against 12% (v/v) 2-methylpentane-2,4-diol (MPD) [7]. Crystals were subsequently transferred to solutions of increasing MPD concentrations up to a final concentration of 25% (v/v). The crystals (space group $P2_12_12$, one dimeric enzyme molecule per asymmetric unit) showed well grown (110) and (001) rhombic forms.

To record polarized spectra by use of a Zeiss MPM 03 UV-visible microspectrophotometer equipped with polarizers, single crystals were mounted in a cell with quartz windows and were oriented with either the (110) or ($1\bar{1}0$) face of the same crystal perpendicular to the incident light and the *c* axis either parallel or perpendicular to the electric vector. On the basis of the polarized spectra recorded on the two perpendicular faces and measurements of the crystal thickness in either direction, the isotropic spectrum was calculated as $(A_x + A_y + A_z)/3$ [8].

Reaction with substrate and binding of azide were obtained by flowing a medium containing the appropriate concentrations of the various reagents through the crystal in the cell.

Removal of type 2 Cu^{2+} was performed according to Avigliano et al. [9]. Crystals were anaerobically incubated for 48 h in 50 mM phosphate buffer, pH 5.2, 25% MPD (v/v), containing 2 mM dimethylglyoxime, 1 mM EDTA, 5 mM ferrocyanide.

3. RESULTS AND DISCUSSION

3.1. *Polarized absorption spectra of crystalline ascorbate oxidase*

The polarized absorption spectra of single crystals of native ascorbate oxidase reported in fig.1A show all the characteristic bands of the oxidized enzyme in solution, with absorption maxima at 330 nm, 610 nm and around 750 nm; the weak band at 445 nm is also clearly detected. The isotropic spectrum calculated from the polarized spectra (fig.1B) is essentially identical in shape to that recorded for the enzyme in solution [3].

The visible region of the spectrum is very similar to that of other proteins containing type 1 Cu^{2+} [10], for two of which the high resolution three-dimensional structure was determined and single crystal spectra were recorded, i.e. plastocyanin, containing a single type 1 Cu^{2+} in a distorted tetrahedral environment formed by S(Cys), S(Met), N(His), N(His) [11], and Cu^{2+}-substituted liver alcohol dehydrogenase (LADH), containing one type 1 Cu^{2+} per monomer in a distorted tetrahedral environment formed by S(Cys), S(Cys), N(His), O(H_2O) (Merli, A. et al., in preparation). For this protein the crystal structure was not directly determined, but there is evidence [12] that the Cu^{2+} maintain the distorted tetrahedral environment present in the native Zn-enzyme.

The studies on plastocyanin have attributed the bands at 606, 750 nm and a hidden band at 560 nm to charge transfer (CT) transitions from cysteine sulfur to copper and the two very weak bands at 428 and 467 nm to CT transitions from methionine and/or histidines [11].

Ascorbate oxidase shows same absorptions as plastocyanin in the region 500–800 nm, but only one band is clearly visible at 445 nm with polarization perpendicular to the *c* axis, similar to the 428 nm band in plastocyanin [11].

Orthorhombic crystals of Cu^{2+}-substituted LADH (space group $C222_1$) exhibit absorption bands, as other blue copper proteins, at 618 and 750 nm and a weak band at 450 nm, all polarized along the *b* axis plus a distinctive band at 375 nm polarized perpendicularly to the *b* axis.

The close similarity of the ascorbate oxidase spectrum with that of plastocyanin, but not with that of Cu^{2+}-substituted LADH, suggests that type 1 Cu^{2+} of ascorbate oxidase have the same ligand environment as copper present in plastocyanin. Moreover, the close resemblance of the polarization ratio of ascorbate oxidase polarized spectra on the face (110) ($A_{\parallel}/A_{\perp}$) shown in fig.1A with that of plastocyanin polarized spectra on the face (011) suggests that the orientation of the Cu^{2+} ligands with respect to crystal axes is similar in the two proteins, provided the appropriate exchange of axes is made.

The band at 330 nm is functionally associated with type 3 Cu^{2+}, an antiferromagnetically coupled Cu^{2+} pair. In the crystal, this band is strongly polarized along the *c* axis, suggesting a precise orientation of the ligand responsible for this transition. No comparison with other crystalline proteins is at the moment possible.

3.2. *Reaction with ascorbate*

The disappearance of all the copper absorption bands of crystalline ascorbate oxidase upon exposure to solutions containing ascorbic acid indicates that the enzyme can be reduced within the time of diffusion of the substrate through the crystal lattice, i.e. about 30 s. Washing the crystals with an oxygenated medium restored the spectrum of the oxidized enzyme. The crystals did not break

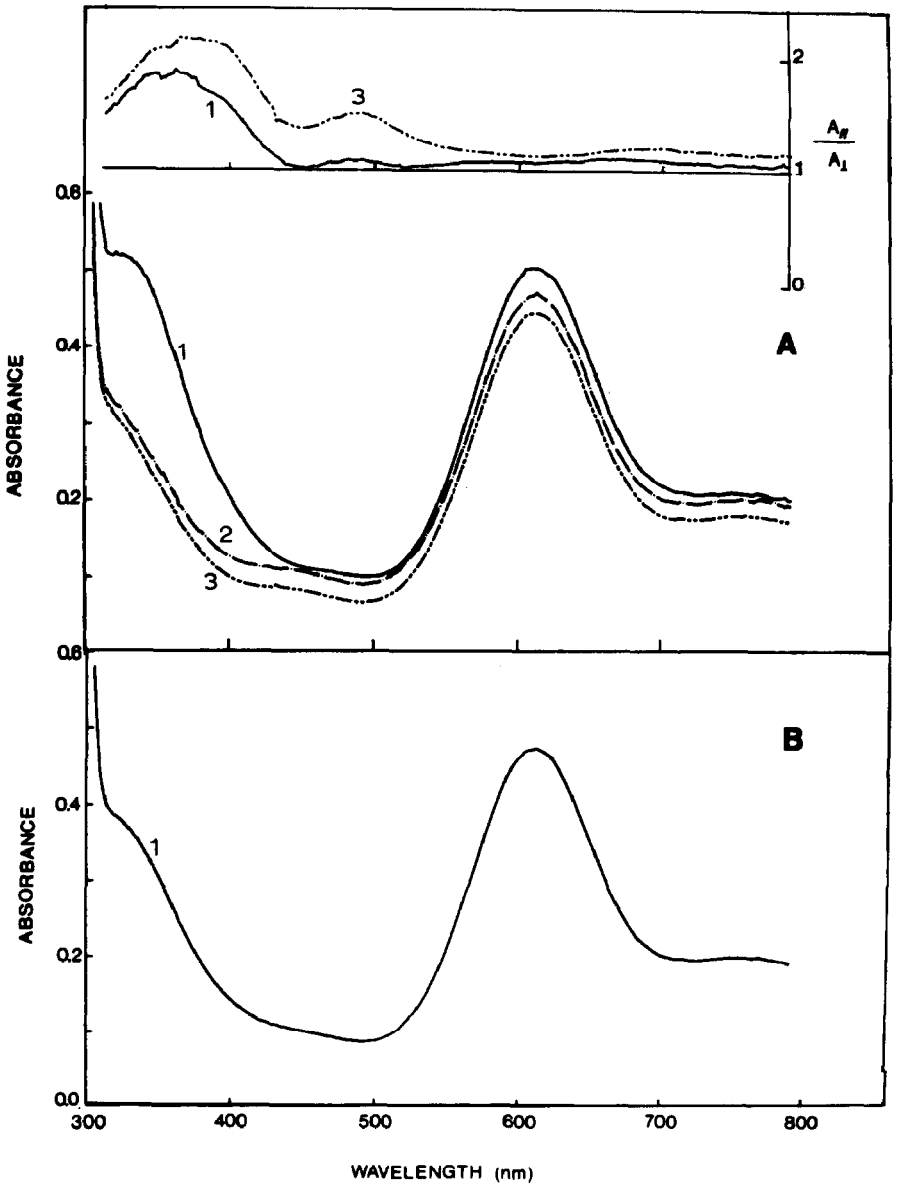

Fig.1. Absorption spectra of single crystals of ascorbate oxidase. (A) Polarized spectra with the electric field vector (E) of the beam of plane polarized light aligned parallel to the c crystal axis (1) or perpendicular to the c crystal axis on the (110) and ($1\bar{1}0$) face of the crystal (2 and 3). The spectra were normalized to a thickness of 0.1 mm. (Inset) Polarization ratio $A_{\parallel}/A_{\perp}$ on the two faces. (B) Crystal isotropic spectrum calculated from the polarized absorption spectra.

or crack upon reaction with ascorbic acid or oxygen. On the basis of these results it can be concluded that crystalline ascorbate oxidase is in an enzymatically active form and that only minor conformational changes occur during the catalytic cycle. However, after attainment of complete reduction, the crystals slowly dissolved (in 10–15 min), unless they were quickly reoxidized.

3.3. *Reaction with azide*

In multicopper oxidases, type 2 Cu^{2+} is the binding site of anions like azide and fluoride [4]. Upon binding of azide to ascorbate oxidase in solution, the absorption of type 3 Cu^{2+} at 330 nm disappears with the concomitant appearance of a band at about 400 nm and a shoulder at 500 nm [4,13]. Similar spectral changes occur in laccase,

whose spectral transitions have been analyzed in detail [14,16]. The absorption at 400 nm was attributed to two CT transitions from azide to type 3 Cu^{2+} in the coupled and uncoupled form. Similarly, the absorption at 500 nm was attributed to the binding of azide to type 2 Cu^{2+}. The parallel increase of these absorptions was interpreted as indicative of the simultaneous binding of one azide molecule to both type 2 and type 3 Cu^{2+}.

In the crystals of ascorbate oxidase, binding of azide causes the disappearance of the band at 330 nm and the appearance of an asymmetric broad band at about 400 nm, polarized parallel to the *c* axis. It is composed of at least two transitions since the polarization ratio is seen to change across the band (fig.2A). In the difference spectrum, a shoulder at 500 nm is clearly resolved. This transition is polarized perpendicularly to the *c* axis of the crystal, with polarization ratio $A_{\parallel}/A_{\perp} = 1.4$ on both (110) and ($1\bar{1}0$) faces of the crystal (fig.2B).

The titration of crystalline ascorbate oxidase with azide shows that, as in solution, the increase of the bands at 400 and 500 nm is concomitant with the decrease of the 330 nm band, with a neat isosbestic point at 352 nm (fig.3). A dissociation constant of the complex of 1.2 mM at pH 5.8 was calculated, similar to that reported in solution [13].

The correspondence of the absorptions of the azide-ascorbate oxidase complex in the crystal and of the azide-laccase complex in solution suggests that in the former as in the latter species azide binds to type 2 and type 3 Cu^{2+}, forming a bridge between two unequivalent copper centers.

The presence of an isosbestic point in the azide titration indicates that the chromophore absorbing at 330 nm is transformed in the course of the reaction.

A broad and weak absorption between 400 and 500 nm at low concentration of azide, indicative of a high-affinity binding site, is reported to be characteristic, in laccase, of a partially reduced state of the trinuclear center formed by the two type 3 and the type 2 Cu^{2+} [14–16]. This absorption was only occasionally detected in ascorbate oxidase crystals.

3.4. *Removal of type 2 Cu^{2+}*

Selective removal of type 2 Cu^{2+} from ascorbate oxidase was obtained in solution by treatment with dimethylglyoxime in the presence of ferrocyanide in anaerobic conditions, with disappearance of the absorption at 330 nm [9]. In the crystal, type 2 Cu^{2+} could be removed under the same conditions used in solution and with the same spectral changes (fig.4). Sinc type 2 Cu^{2+} does not show appreciable optical absorption, in the crystal as in solution indirect evidence of its removal is offered by the disappearance of the 330 nm band. Further evidence is provided by the failure to observe azide binding in these enzyme crystals.

After addition of hydrogen peroxide to the dimethylglyoxime-treated crystals the absorption

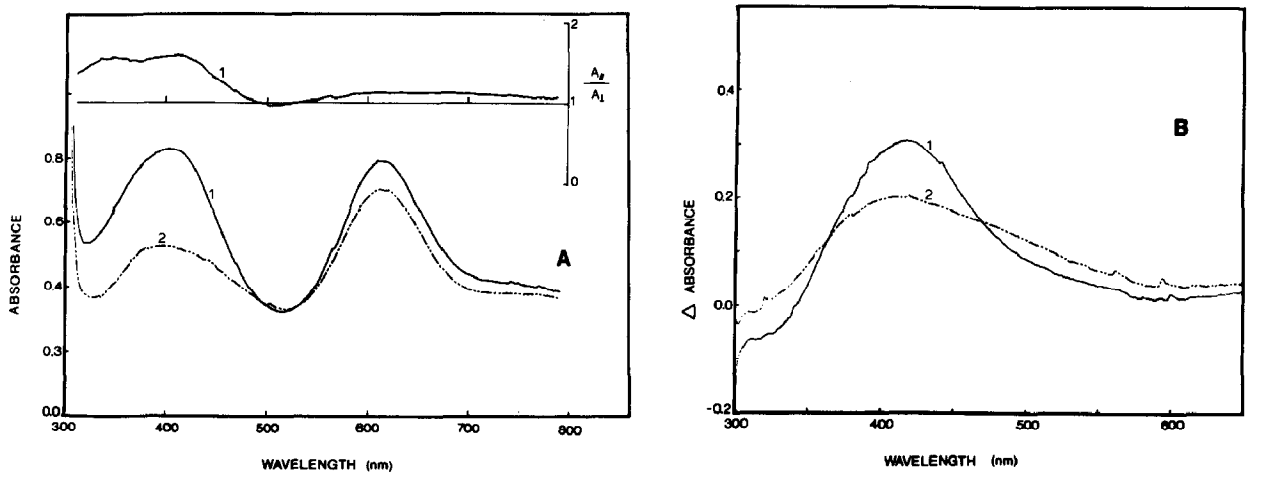

Fig.2. Polarized single crystal absorption spectra of the complex of ascorbate oxidase with azide. (A) Polarized spectra in the presence of 25 mM NaN_3; (1) $E_{\parallel}c$; (2) $E_{\perp}c$. (B) Absorption difference spectra (1) $E_{\parallel}c$; (2) $E_{\perp}c$.

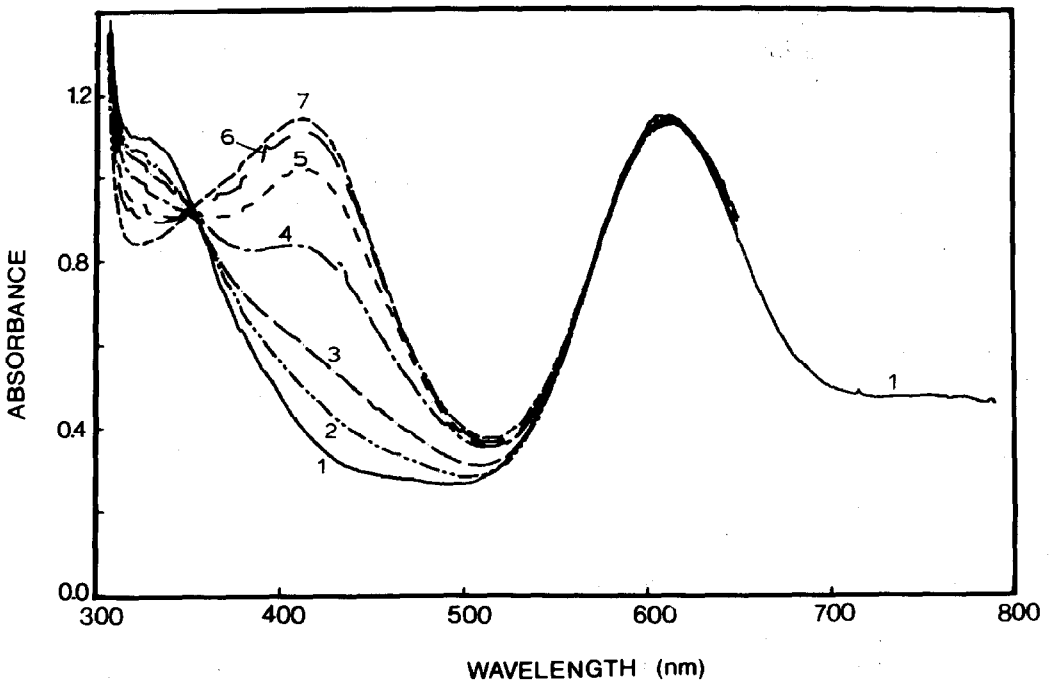

Fig.3. Titration of ascorbate oxidase with azide. Spectra were recorded with $E_{\parallel}$c. (1) native enzyme in 50 mM phosphate, pH 5.8, 25% MPD. Azide concentrations: (2) 0.25 mM; (3) 1.0 mM; (4) 1.5 mM; (5) 2.5 mM; (6) 5.0 mM; (7) 15 mM.

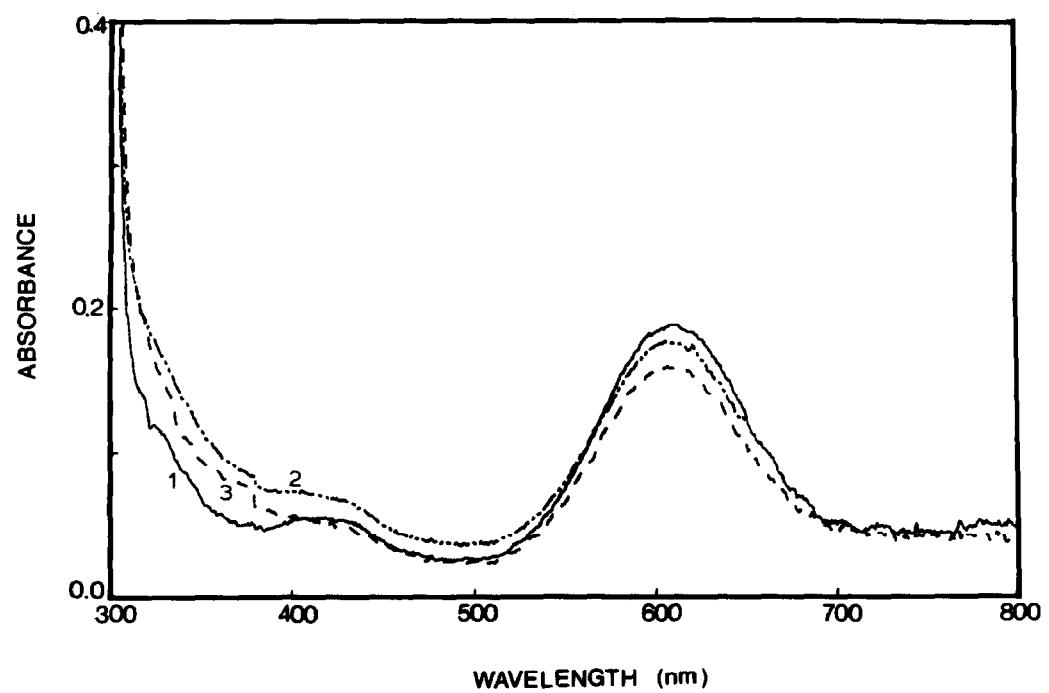

Fig.4. Absorption spectra of type Cu^{2+}-depleted crystalline ascorbate oxidase recorded with $E_{\parallel}$c. (1) the enzyme after treatment with dimethylglyoxime; (2) the type 2 Cu^{2+}-depleted crystalline ascorbate oxidase after addition of 20 mM H_2O_2; (3) the type 2 Cu^{2+}-depleted crystalline ascorbate oxidase after addition of 25 mM NaN_3.

at 330 nm increases to about 50% of the initial value, indicating that reoxidation of type 3 Cu^{2+} does not occur as extensively as in laccase in solution [17]. Even after partial oxidation, azide binding is not observed.

The possibility to remove type 2 Cu^{2+} from crystalline enzyme is relevant to the identification of this copper species in the electron density map.

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