PRELIMINARY CRYSTALLOGRAPHIC STUDY OF ASCORBIC ACID OXIDASE FROM GREEN ZUCCHINI SQUASH

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1. Introduction

The copper protein ascorbic acid oxidase (EC 1.10.3.3), which can be found in a broad variety of plants, catalyzes the 4-electron reduction of dioxygen to water, utilizing preferentially L-ascorbic acid as electron donor. EPR studies have provided evidence that the first step of the reaction sequence is the removal of a single electron from ascorbic acid giving a free radical of the reducing substrate [1]. In solution ascorbic acid oxidase appears to be a dimer (mol. wt 140 000), probably consisting of identical subunits [2]. The dimeric molecule contains 8 Cu^{2+} [3] present in at least 3 different states classified as type 1 type 2 (both paramagnetic) and diamagnetic type 3 copper [4]. Cu^{2+} (type 1) is associated with an absorption band around $\lambda = 610$ nm and is responsible for the deep blue colour of this 'blue oxidase'.

Since a structural analysis of ascorbic acid oxidase would reveal a fascinating coordination chemistry of copper in a biological macromolecule and could provide new information concerning the mechanism of electron transport, subunit composition, distribution and binding of copper, we have crystallized ascorbic acid oxidase and now present preliminary crystallographic data.

2. Experimental

Ascorbic acid oxidase was prepared from green zucchini squash *(Cucurbita pepo medullosa)* by an improved 6 step procedure, including two chromatographic and one electrophoretic purification steps. Since this procedure has been detailed in [5] we report here essential data of the purified enzyme only: copper content, 3.8 μg Cu/mg protein, corresponding to ~8.5 Cu atoms/mol enzyme based on mol. wt 140 000: $A_{330}/A_{610} = 0.85$, $A_{610}/A_{500} = 5.0$; the preparation was spec. act. 3600 U/mg.

With the aim of growing crystals suitable for X-ray analysis, we surveyed a variety of different combinations of protein concentrations, buffer systems and stabilizing additives (L-ascorbic acid, citric acid) by the vapour diffusion method at 20°C and 4°C.

X-ray precession and rotation photographs were recorded on Nonius-Enraf cameras in a cold room at 4°C. The X-ray source was a Philips fine focus X-ray tube operated at 1.5 kW with a focal spot size of 0.4 × 4.0 mm. Graphite monochromatized CuK_α radiation was used throughout. The crystal to focus distance was 300 mm. Exposure ranged from 50–120 h.

3. Results and discussion

The best crystals could be grown at 4°C with 1.9 M sodium-potassium phosphate buffer (pH 7.0) as the reservoir solution. The initial droplet contained ascorbic acid oxidase in a solution of 1.0 M phosphate buffer. After equilibrium has been established in the diffusion chambers the final protein concentration was 5–6 mg/ml. Crystals of ascorbic acid oxidase are deep blue and show a very uncommon shape. They grew to full size (about 0.4 × 0.2 × 0.1 mm) in 6–8 weeks. With >8 mg protein/ml we obtained

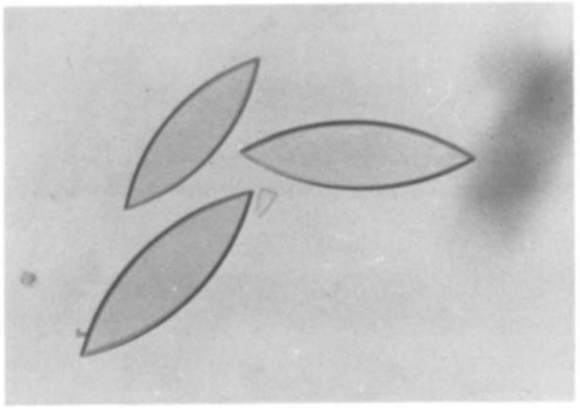

Fig.1. Photomicrograph of an orthorhombic ascorbic acid oxidase crystal grown from 1.9 M phosphate buffer, (pH 7.0).

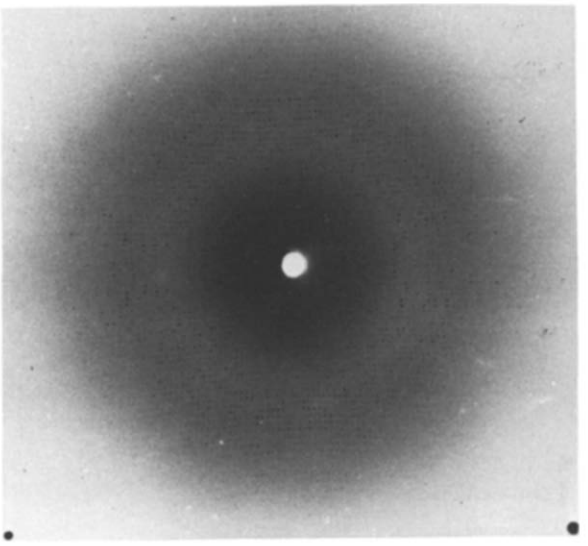

Fig.2. Rotation photograph of an ascorbic acid oxidase crystal: *Nkl* levels; $\Delta\theta = 2°$; 20 cm pinhole collimator, diam. 0.4 mm; crystal to film distance, 75 mm; exposure time, 100 h; X-ray tube, $U = 50$ kV, $J = 30$ mA.

much bigger crystals, but unfortunately a strong tendency of twinning could be observed, possibly induced upon enhancement of the growth rate. The crystals are rather sensitive to the mechanical manipulations necessary to seal them in X-ray glass capillaries. Figure 1 shows a photomicrograph of an ascorbic acid oxidase crystal.

X-ray photographs show an orthorhombic unit cell with dimensions of: $a = 190.7$ Å; $b = 125.2$ Å; $c = 112.3$ Å; $\alpha=\beta=\gamma= 90°$. 10° precession photographs of the *hk*0 and the 0*kl* levels of the reciprocal lattice indicate mm symmetry, demonstrating point group 222. Systematic absences of reflexions were observed for $h = 2n + 1$, $k = 2n + 1$, $l = 2n + 1$, that one characteristic of 3 2-fold screw axes. Based on these findings the space group was established as $P2_12_12_1$ with 4 general positions. On 'still' photographs the reflexions extend to a resolution of ~2.5 Å. Figure 2 shows a rotation photograph of an ascorbic acid oxidase crystal.

With the information that ascorbic acid oxidase is a dimer of mol. wt 140 000 one can determine the number of molecules in the asymmetric unit of the crystal cell and thus assess the magnitude of problems posed in an X-ray structure analysis. Assuming 2 dimers/asymmetric unit, a packing density of $V_M = 2.4$ Å^3/dalton can be obtained, a value fitting well into the range found with other protein crystals [6]. Implying that they are identical, the monomers obviously should be related by local symmetry elements. Rotational and translational search techniques applied to the Patterson map from native intensity data certainly will be capable of revealing these symmetry relations. During structure analysis of ascorbic acid oxidase with mol. wt 280 000 in the asymmetric unit of the crystal cell severe problems may arise, especially in finding good heavy atom derivatives and in the determination of heavy atom positions.

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