Crystallization and preliminary X-ray diffraction studies of the iron superoxide dismutase from the eukaryote *Vigna unguiculata*

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Synopsis: The iron superoxide dismutase from an eukaryote, the leguminous plant *Vigna unguiculata*, has been crystallized for the first time. At cryogenic temperatures, monoclinic crystals diffract to a resolution limit of 1.80 Å using synchrotron radiation.

Abstract

Superoxide dismutases are a family of metalloenzymes that catalyze the dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide, and thus represent a primary line of defence against oxidative stress. The iron-containing superoxide dismutases are only found in prokaryotes and plants. The iron superoxide dismutase of *Vigna unguiculata* (cowpea) consists of two polypeptides of 27 kDa, each binding an iron atom as cofactor. The protein was cloned, overexpressed in *Escherichia coli*, and purified. After several refined screenings, crystals suitable for X-ray diffraction analysis were obtained. The crystals belong to the monoclinic space group C2, with unit cell parameters $a=82.54$ Å, $b=48.41$ Å, $c=64.28$ Å, $\alpha=\gamma=90^\circ$, $\beta=119.66^\circ$, and contain one molecule per asymmetric unit. At cryogenic temperatures the crystals diffracted to a resolution limit of 1.80 Å using synchrotron radiation at the European Synchrotron Radiation Facility.

1. Introduction

Reactive oxygen species, such as the superoxide radicals and hydrogen peroxide, are inevitably generated as by-products in important processes involving electron transport, including respiration, photosynthesis, and nitrogen fixation (Dalton, 1995). Although the superoxide radicals are not especially toxic by themselves, they can act as precursors of the hydroxyl radicals, which can oxidize virtually all types of cellular components (Halliwell & Gutteridge, 1999). Because there are no enzymatic mechanisms to eliminate the hydroxyl radicals, it is not surprising that all organisms, with a few exceptions, contain superoxide dismutases (SODs). These are a family of metalloenzymes that catalyze the dismutation of superoxide radicals into oxygen and hydrogen peroxide, and, in so doing, avoid the risk of formation of hydroxyl radicals. Three main classes of SODs can be distinguished based on the metals at the catalytic active site. Typically, the CuZnSODs are localized in the cytosol and chloroplasts, the MnSODs in the mitochondria and peroxisomes, and the FeSODs in the chloroplasts (Sandalio *et al.*, 1987; Kanematsu & Asada, 1990; Van Camp *et al.*, 1990). The FeSODs are the least studied group of SODs and can be found in prokaryotes and plants, but not in animals. In higher plants, FeSODs play a protective role against the free radicals originated in light-mediated processes such as photosynthesis and photoinhibition (Tsang *et al.*, 1991). Very recently, additional functions have been suggested for FeSODs in cellular protection against the oxidative stress induced by heavy metals in bacteria and dinoflagellates (Geslin *et al.*, 2001; Okamoto *et al.*, 2001) or taking place during senescence of legume nodules (Moran *et al.*, 2003).

We have recently characterized the FeSOD from nodules and leaves of the leguminous plant, *Vigna unguiculata* (cowpea). The enzyme is a homodimer of approximately 57 kD and, contrary to other higher plant FeSODs, is localized in the cytosol (Moran *et al.*, 2003). In this work, high amounts of recombinant protein have been produced in soluble form in *Escherichia coli* and used to obtain crystals and preliminary X-ray diffraction data. Several structures of prokaryotic FeSODs have been determined by X-ray crystallography (ie. Stallings *et al.*, 1983; Cooper *et al.*, 1995;

Bond *et al.*, 2000). To our knowledge, however, this is the first report analyzing the structure of an eukaryotic FeSOD.

2.Materials and methods

Expression and purification

The cDNA encoding *V. unguiculata* FeSOD (VuFeSOD), without the transit peptide, was cloned into a pET28a(+) vector (Novagen, Madison, WI) and finally used to transform *E. coli* BL21(DE3) cells. These were grown at 37°C in Luria-Bertani broth containing kanamycin (100 µg/mL) until the culture reached an optical density of 0.5. Then, protein expression was induced by addition of 0.5 mM isopropyl β-thiogalactoside for 3 h. After induction the cells were pelleted by centrifugation and stored at -80°C until used. Protein purification was performed essentially as described in Moran *et al.* (2003). The cell paste (1.5 g) was resuspended in 50 mM Tris-500 mM NaCl (pH 8.0), and then sonicated and subjected to chromatography onto a HiTrap Chelating column (Amersham Biosciences), following the manufacturer instructions. The collected fractions were digested with 5 units of thrombin for 24 h at 4°C, dialysed and subjected to chromatography onto a DEAE (DE-52, Whatman) column (7.5 x 1.5 cm). The column was previously equilibrated with 10 mM phosphate buffer pH 7.8, and the sample was eluted with 100 mM phosphate buffer (pH 7.8). Finally, The protein-containing fractions were pooled and the buffer was exchanged to 50 mM Tris-HCl (pH 7.8) using a PD-10 desalting column (Amersham Biosciences). The protein homogeneity and purity was checked on SDS, native and IEF gels, with optimal results. Protein concentration was determined by a dye-binding assay (Bio-Rad) using BSA as a standard.

Crystallisation

Highly pure and active FeSOD was concentrated to 4 mg/ml, the protein preparation showed a light yellow colour indicating the presence of Fe. Crystallisation trials were performed using the hanging-drop vapour-diffusion method at 277 K. Initially small crystals and needles were obtained after 48 h using the screen I and II from Hampton Research (CA, USA). The optimisation of the crystallisation conditions was consequently necessary. The improvement of the crystal quality and size was achieved by refining the initial crystallisation conditions and the use of microseeding. The new

crystals grew from a single nucleation site, and were thicker than the previous ones. FeSOD gave different types of crystals during this screening with different conditions (Fig.1). Small hexagonal plates grew in 200 mM magnesium chloride hexahydrate, 100 mM Tris-HCl (pH 8.5), 30% w/v polyethylene glycol 4000 (Fig.1a) with approximate maximum dimensions of 50 x 50 x 20 μm . Furthermore, small orthorhombic crystals (Fig.1b) were also obtained using 100 mM trisodium citrate dihydrate (pH 5.6), 20% (w/v) polyethylene glycol 4000 and 20% (v/v) isopropanol, as mother liquor with approximate maximum dimensions of 60 x 30 x 40 μm . Crystals with rhombohedral shape (Fig 1c) were grown using the same crystallisation conditions with a bigger size of approximately, 90 x 90 x 10 μm . Finally the monoclinic crystals with size of 60 x 20 x 30 μm (Fig. 1d), which grew in 200 mM ammonium sulphate, 100 mM sodium acetate (pH 5.0) and 25 % (w/v) polyethylene glycol 4000 were the best diffracting ones and therefore used for data collection.

Data collection and reduction

The monoclinic crystals (Fig. 1d) were soaked during 10 to 20 seconds in a cryoprotectant solution composed of 200 mM ammonium sulphate, 100 mM sodium acetate (pH 5.0) and 35 % (w/v) polyethylene glycol 4000, mounted in a loop and immediately flash-cooled to 100 K. A complete data set to 1.80 Å was collected from a single crystal, using synchrotron radiation at the beamline BM14S at ESRF, Grenoble. Diffraction data were recorded on a MARCCD detector with a diameter of 130 mm. The crystal to detector distance was 100 mm, $\Delta\phi=1^\circ$, and the exposure time 120 seconds. 160 images were collected in total at a wavelength of 1.033 Å. Processing and scaling were accomplished with MOSFLM (Leslie, 1992) and SCALA (Evans, 1993) in the CCP4 suite (CCP4 suite, Collaborative Computational Project Number 4, 1994). Statistics for the crystallographic data are summarised in Table I.

3. Results and discussion

The recombinant enzyme FeSOD from *V. unguiculata* was expressed routinely in *E. coli* with a yield of around 8 mg per litre of culture. The protein was isolated using a hexa-his-tag at the NH₂ terminus, which was removed by thrombin for crystallisation. Several types of crystals were obtained and those that diffracted optimally after freezing were used for data collection. Data were collected through a 160° rotation in ϕ . The

crystals belong to the space group C2 with unit cell parameters $a=82.54 \text{ \AA}$, $b=48.41 \text{ \AA}$, $c=64.28 \text{ \AA}$, $\alpha=\gamma=90^\circ$, $\beta=119.66^\circ$ and a unit cell volume of $223195 \text{ \AA}^3$. The Matthews coefficient of $2.23 \text{ \AA}^3 \text{ Da}^{-1}$ suggests one protein molecule per asymmetric unit and a solvent content of 44.46 %. Probably due to the crystal size, no good data sets could be collected using an in-house source. Therefore the cryobuffer selection and the first diffraction trials were performed using a synchrotron radiation source. Although small crystals were easily obtained, several steps of seeding were necessary in order to obtain reasonable sized crystals for data collection. Reproducibility was purification dependent and some purification batches did not yield crystals. Finally several native data sets were collected at 100 K on beamline BM14S at the European Synchrotron Radiation Facility (ESRF) in Grenoble. Synchrotron radiation increased the average resolution to $1.80 \text{ \AA}$. Statistics of the best data set is shown in table I. In order to solve the phase problem we collected a data set at the anomalous peak of iron ($1.731 \text{ \AA}$). Previously we recorded a Xanes scan using a mounted crystal. Although the scan was noisy, a clear signal at the Fe edge was observed. After inspection of the data no anomalous signal could be detected and the anomalous Patterson spectrum did not yield useful information to locate the Fe atom. Unfortunately this data set was not good enough to help us to solve the structure. Both molecular replacement and selenomethionine production for MAD are underway to solve the phase problem. We hope that the first structure for an eukaryotic FeSOD, along with biochemical and genetic data, provides further insights on the antioxidative role of this rather enigmatic type of SOD.

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Table I

Data collection statistics

Resolution limits (Å)	$\langle I/\sigma(I) \rangle$	Completeness (%)	Multiplicity	Observed Reflections	Unique Reflections	R _{sym}
30.0-5.69	9.1	99.1	3.3	2230	686	0.044
5.69-4.03	15.7	99.7	3.4	4057	1205	0.039
4.03-3.29	12.8	99.8	3.4	5320	1569	0.051
3.29-2.85	9.5	99.9	3.4	6211	1824	0.073
2.85-2.55	5.2	99.9	3.4	7025	2079	0.116
2.55-2.32	4.5	99.9	3.4	7742	2291	0.164
2.32-2.15	3.2	99.9	3.4	8281	2471	0.221
2.15-2.01	2.5	100.0	3.3	8804	2658	0.295
2.01-1.90	1.5	100.0	3.3	9485	2836	0.467
1.90-1.80	0.9	99.0	3.1	8653	2776	0.778
Overall	6.2	99.0	3.3	67808	20395	0.101

$$R_{\text{sym}} = \sum_{\eta} \sum_i |I_{\eta,i} - \langle I_{\eta} \rangle| / \sum_{\eta} \sum_i |I_{\eta,i}|$$

Figure Legends

Figure 1.- Different types of crystals from FeSOD were grown at 277 K. a) Hexagonal crystals. b) Orthorhombic crystals. c) Rhombohedral crystals. d) Monoclinic crystals of FeSOD from *V. unguiculata* which behaved well with the cryobuffer conditions described in the text and diffracted to 1.80 Å. (See the results and discussion section for details). Pictures have been taken at different magnification settings.

Figure 2.- a) Diffraction pattern from the native crystals using synchrotron radiation on the beamline BM14S at the ESRF. b) Detailed picture showing some spots at the edge of the plate, which is at a resolution of 1.80 Å.

Figure 1

Figure 2a

Figure 2b