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Crystallization and preliminary X-ray diffraction studies of the eukaryotic iron superoxide dismutase (FeSOD) from *Vigna unguiculata*

Inés G. Muñoz,[a] José Fernando Moran,[b] Manuel Becana[b] and Guillermo Montoya[a]*

[a]Structural Biology and Biocomputing Programme, Spanish National Cancer Center (CNIO) Macromolecular Crystallography Group, c/Melchor Fdez. Almagro, 3 28029 Madrid, Spain, and [b]Estación Experimental de Aula Dei Consejo Superior de Investigaciones Científicas (CSIC), Avda. Montañana, 1005 50059 Zaragoza, Spain

Correspondence e-mail: gmontoya@cnio.es

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Eukaryotic iron superoxide dismutases (FeSODs) are homodimeric proteins that constitute a fundamental protection against free radicals, which can damage essential cellular mechanisms. The protein was cloned and overexpressed in *Escherichia coli* with an N-terminal His tag. Crystallization experiments of the protein resulted, after several refined screenings, in crystals suitable for X-ray diffraction analysis. The crystals belong to the monoclinic space group $C2$, with unit-cell parameters $a = 82.54$, $b = 48.41$, $c = 64.28$ Å, $\alpha = \gamma = 90$, $\beta = 119.66^\circ$, 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 (ESRF).

1. Introduction

Reactive oxygen species, such as superoxide radicals and hydrogen peroxide, originate as byproducts in important metabolic processes involving electron transport, such as respiration, photosynthesis or symbiotic nitrogen fixation. Although superoxide radicals are not especially toxic in themselves, they can act as precursors of hydroxyl radicals, which can react with a wide variety of molecular targets in the cell, producing oxidative damage that can lead to genetic mutation or cell death (Halliwell & Gutteridge, 1999). Living organisms have developed direct or indirect mechanisms to protect their cellular machinery against oxidative damage. A large number of small molecules, such as vitamin E, ascorbic acid or gluthatione, have been shown to interact with free radicals, showing antioxidant and anticarcinogenic properties (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; EC 1.15.1.1; Gutteridge & Halliwell, 2000). This is a family of metalloenzymes that catalyze the dismutation of superoxide radicals into oxygen and hydrogen peroxide and, in so doing, avoid the risk of the formation of hydroxyl radicals.

Three main classes of SODs can be distinguished based on the metals at the catalytic active site. Typically, CuZnSODs, which contain both cations in the active site, are localized in the cytosol and chloroplasts, MnSODs in the mitochondria and peroxisomes and FeSODs in the chloroplasts (Fridovich, 1995; Sandalio *et al.*, 1987; Kanematsu & Asada, 1990; Van Camp *et al.*, 1990). The FeSODs are the least studied group of SODs and in the higher organisms appear to be present exclusively in plants, where they play a protective role against the free radicals originating from light-mediated processes such as photosynthesis and photoinhibition (Tsang *et al.*, 1991). While CuZnSOD is a primarily eukaryotic enzyme found in animal and plant tissues, MnSODs and FeSODs are structurally related families found in many prokaryotic organisms, from where they have differentially evolved towards more complex forms in higher organisms. Studies of SOD null-mutant phenotypes in bacteria have revealed that, despite their common enzymatic function, they show a broad spectrum of biological roles (Touati, 2002). FeSOD is a typical protective enzyme during light photosynthetic processes and has been found in several cyanobacteria, in algae (Okamoto *et al.*, 2001) and in a wide variety of higher plants (Kliebenstein *et al.*, 1998).

In addition to the photosynthesis protective role of the eukaryotic FeSOD, new functions have been suggested as a defensive enzyme for the nitrogenase in the heterocysts of *Anabaena* (Li *et al.*, 2002) and acting against metal toxicity in bacteria and dinoflagellates (Okamoto *et al.*, 2001) or in senescent legume nodules (Moran *et al.*, 2003). Complete cDNA clones encoding enzymes of each type have been isolated and used to construct transgenic plants overexpressing SODs. These transgenic plants with higher levels of these antioxidant genes

can have additional advantages owing to the protective role played by these enzymes (Van Breusegem *et al.*, 1999); McKersie *et al.*, 2000; Rubio *et al.*, 2001).

Several FeSOD structures have been solved, all of them from non-photosynthetic eubacteria or archaea (Stoddard, Howell *et al.*, 1990; Stoddard, Ringe *et al.*, 1990; Lah *et al.*, 1995; Cooper *et al.*, 1995; Stallings *et al.*, 1983; Bond *et al.*, 2000), but to our knowledge no eukaryotic FeSOD structure has yet been solved. Here, we describe the first crystals of an eukaryotic FeSOD from the plant *Vigna unguiculata* (cowpea). We also report the expression, purification, crystallization and preliminary X-ray characterization of the protein crystals.

2. Materials and methods

2.1. Expression and purification

The cDNA encoding *V. unguiculata* FeSOD (VuFeSOD), without the transit peptide, was cloned into a pET28a(+) vector (Novagen, Madison, WI, USA) and finally used to transform the *Escherichia coli* BL21(DE3) line. *E. coli* cells containing the pET28a(+):FeSOD construct were grown at 319 K in Luria–Bertani broth containing kanamycin (100 µg ml^{-1}). When the culture reached an OD of 0.5, protein expression was induced with 0.5 m*M* isopropyl β-thiogalactoside (IPTG) for 3 h. After induction, the cells were pelleted by centrifugation and stored at 193 K until use.

Protein purification was performed essentially as described in Moran *et al.* (2003). The cell paste (1.5 g) was resuspended in 50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl buffer and then sonicated and subjected to chromatography on a HiTrap Chelating column (Amersham Biosciences) following the manufacturer's instructions. The collected fractions were digested with 5 units of thrombin for 24 h at 277 K, dialysed and subjected to chromatography on a DEAE (DE-52, Whatman) column (7.5 × 1.5 cm). The column was previously equilibrated with 10 m*M* phosphate buffer pH 7.8 and the sample was eluted with 100 m*M* phosphate buffer pH 7.8. Finally, the protein-containing fractions were pooled and the buffer was exchanged to 50 m*M* Tris–HCl pH 7.8 using a PD-10 desalting column (Amersham Biosciences). The protein homogeneity and purity were checked with an SDS–PAGE (15%) gel, a native PAGE (15%) gel and an IEF gel, with optimal results. The protein concentration was determined by a dye-binding assay (Bio-Rad) using BSA as a standard.

2.2. Crystallization

Highly pure and active FeSOD was concentrated to 4 mg ml^{-1}; the protein preparation showed a light yellow colour indicating the presence of Fe. Crystallization 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 Crystal Screens I and II from Hampton Research (CA, USA). Optimization of the crystallization conditions was thus necessary. Improvement of the crystal quality and size was achieved by refining the initial crystallization conditions and by 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 of different conditions (Fig. 1). Small hexagonal plates grew in 200 m*M* magnesium chloride hexahydrate, 100 m*M* Tris hydrochloride pH 8.5, 30%(*w*/*v*) polyethylene glycol 4000 (Fig. 1*a*) with approximate maximum dimensions of 50 × 50 × 20 µm. Small orthorhombic crystals (Fig. 1*b*) were also obtained using 100 m*M* trisodium citrate dihydrate pH 5.6, 20%(*w*/*v*) polyethylene glycol 4000 and 20%(*v*/*v*) 2-propanol as mother liquor, with approximate maximum dimensions of 60 × 30 × 40 µm. Crystals of rhombohedral shape (Fig. 1*c*) were grown using the same crystallization conditions and had a larger size of approximately 90 × 90 × 10 µm. Finally, monoclinic crystals with dimensions 60 × 20 × 30 µm (Fig. 1*d*), which grew in 200 m*M* ammonium sulfate, 100 m*M* sodium acetate pH 5.0 and 25%(*w*/*v*) polyethylene glycol 4000, were the best-diffracting crystals and were therefore used for data collection.

2.3. Data collection and reduction

The monoclinic crystals (Fig. 1*d*) were soaked for 10–20 s in a cryoprotectant solution composed of 200 m*M* ammonium sulfate, 100 m*M* 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 beamline BM14S, ESRF, Grenoble. Diffraction data were recorded on a MAR CCD detector with a diameter of 130 mm. The crystal-to-detector distance was 100 mm, $\Delta\varphi = 1^\circ$ and the exposure time was 120 s. A total of 160 images were collected at a wavelength of 1.033 Å. Processing and scaling were accomplished with *MOSFLM* (Leslie, 1992) and *SCALA* (Evans, 1993) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Statistics of the crystallographic data are summarized in Table 1.

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 *E. coli* culture. The protein was isolated using a hexa-His tag at the N-terminus,

Table 1
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

† $R_{sym} = \sum_h \sum_i |I_{h,i} - \langle I_h\rangle| / \sum_h \sum_i |I_{h,i}|$.

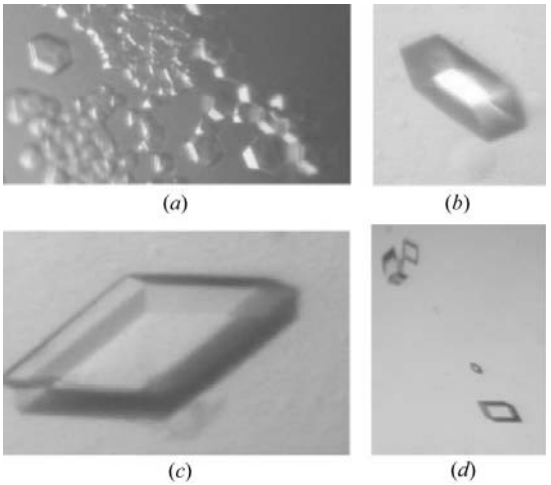
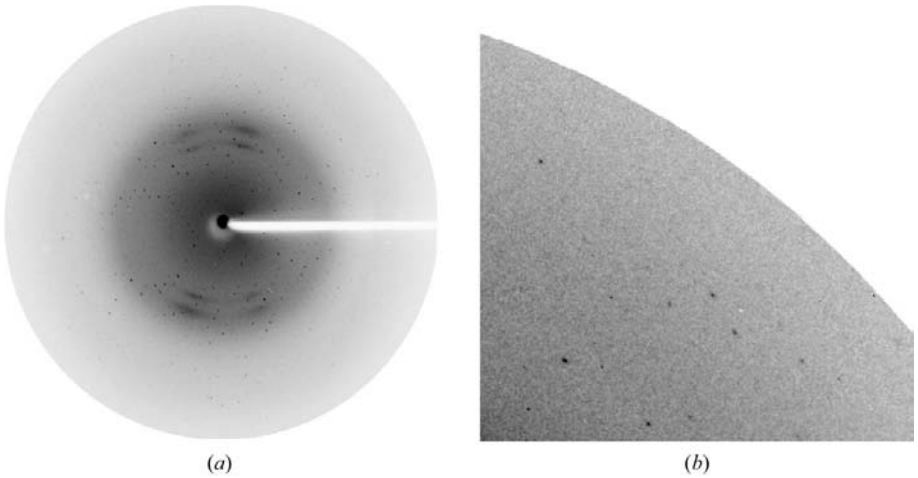


Figure 1
Different types of crystals from FeSOD grown at 277 K. (*a*) Hexagonal crystals. (*b*) Orthorhombic crystals. (*c*) Rhombohedral crystals. (*d*) Monoclinic crystals of FeSOD from *V. unguiculata* which behaved well with the cryobuffer conditions described in the text and diffracted to 1.80 Å (see §3 for details). The pictures were taken at different magnification settings.



(*a*) (*b*)

Figure 2
(*a*) Diffraction pattern from the native crystals using synchrotron radiation on 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.

which was removed with the help of thrombin for crystallization. Several types of crystals were obtained and those that diffracted optimally after freezing were used for data collection. Data were collected over a 160° rotation in φ. The crystals belong to space group $C2$, with unit-cell parameters $a = 82.54$, $b = 48.41$, $c = 64.28$ Å, $\alpha = \gamma = 90$, $\beta = 119.66°$ and a unit-cell volume of 223 195 Å^3. The Matthews coefficient of 2.23 Å^3 Da^{-1} suggests one protein molecule per asymmetric unit and a solvent content of 44.46%. Probably because of the crystal size, no good data sets could be collected using an in-house source. Therefore, a cryobuffer was selected and initial diffraction trials were performed using a synchrotron-radiation source. Although small crystals were easily obtained, several seeding steps were necessary in order to obtain crystals of a reasonable size 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 (Fig. 2). Synchrotron radiation increased the average resolution to 1.80 Å. The statistics of the best data set are shown in Table 1.

In order to solve the phase problem, we collected a data set at the anomalous peak of iron (1.731 Å). A XANES scan had previously been recorded 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 did not yield information useful for location of the Fe atom. Unfortunately, this data set was not good enough to help us to solve the structure. Both molecular replacement and selenomethionine-derivative production for MAD are under way in order to solve the phase problem. We hope that the structure of the first eukaryotic FeSOD from higher plants in combination with our biochemical and genetic experiments will provide details on the molecular level of the protective mechanisms against oxidative damage and reveal differences from the prokaryotic system.

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