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Expression, purification and crystallization of MnSOD from *Arabidopsis thaliana*

Manganese superoxide dismutase (MnSOD) is an essential primary antioxidant enzyme. MnSOD plays an important role in plant tolerance to abiotic stress and is a target candidate for increasing stress tolerance in crop plants. Although the structure and kinetic parameters of MnSODs from several organisms have been determined, this information is still lacking for plant MnSODs. Here, recombinant MnSOD from *Arabidopsis thaliana* (AtMnSOD) was expressed, purified and crystallized. A nearly complete data set could only be obtained when a total rotation range of 180° was imposed during data collection, despite the seemingly tetragonal metric of the AtMnSOD crystal diffraction. The data set extended to 1.95 Å resolution and the crystal belonged to space group *P*1. Molecular-replacement calculations using an ensemble of homologous SOD structures as a search model gave a unique and unambiguous solution corresponding to eight molecules in the asymmetric unit. Structural and kinetic analysis of AtMnSOD is currently being undertaken.

1. Introduction

Superoxide dismutases (SODs) are a family of metalloenzymes that protect cells from the harmful effects of superoxide radical by catalyzing its dismutation into molecular oxygen and hydrogen peroxide (Fridovich, 1995). Depending on the metal in their active site, SODs are classified into four groups: Cu/ZnSODs, NiSODs, FeSODs and MnSODs. Each SOD group displays a distinct subcellular distribution and structural features (Zelko *et al.*, 2002; Miller, 2012).

MnSOD is present in the cytoplasm of prokaryotes and is mainly localized in the mitochondria of eukaryotic cells (Miller, 2012). The crystal structures of several MnSODs have been determined. While most of the bacterial proteins form dimers, the eukaryotic proteins are generally homotetramers. The MnSOD reaction mechanism has been investigated in a few species, mainly through pulse-radiolysis studies (Abreu & Cabelli, 2010). These studies have shown that MnSOD catalyzes the dismutation of superoxide through a complex mechanism involving product inhibition. The rate of product inhibition varies in MnSODs from different organisms, defining the concentration of superoxide at which the SOD activity starts diminishing (Abreu & Cabelli, 2010; Sheng *et al.*, 2011).

In plants, MnSOD plays important roles in response to environmental stress, as demonstrated by transgenic overexpression of the MnSOD gene, which results in enhanced tolerance to salt and drought stresses (Wang *et al.*, 2005, 2007). Therefore, the MnSOD gene can be seen as a candidate for increasing stress tolerance in crop plants (Abreu *et al.*, 2013; Gill & Tuteja, 2010). MnSOD has also been reported to play important roles in plant growth (Morgan *et al.*, 2008) and in maintaining reactive oxygen species (ROS) homeostasis during embryo-sac development (Martin *et al.*, 2013). However, detailed information regarding the structure and kinetic mechanism of plant MnSODs is still lacking. Here, we describe the expression, purification, crystallization and preliminary X-ray diffraction analysis of MnSOD from the model plant *Arabidopsis thaliana* (AtMnSOD). This work represents a step towards the elucidation of the first crystal structure of a plant MnSOD.

2. Materials and methods

2.1. Cloning, expression and purification

The coding sequence for AtMnSOD, excluding its mitochondrial target signal peptide, was amplified from *Arabidopsis* cDNA using primers containing the Gateway adapters *attB1* and *attB2* (F1, GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACTTTTACGCTTCTGATCTTC, and R1, GGGGACCACTTTGTACAAGAAAGCTGGGTCGATTCAAGTTGTTTCCTTCTCATAA). The approximately 760 bp fragment obtained was then cloned into pDONR221 (Gateway system) and sequenced to assure that no mistakes were introduced into the nucleotide sequence by the polymerase chain reaction. The AtMnSOD coding sequence was then transferred into pDEST17 using the LR clonase reaction (Gateway system). The resulting pDEST17-AtMnSOD was sequenced and subsequently transformed into *Escherichia coli* Rosetta pLysS cells (Novagen) for protein expression. Cells were grown at 310 K in 1 l M9 minimal medium. When the OD₆₀₀ reached 0.6, MnCl₂ was added to the culture cells to a final concentration of 0.4 mM and protein expression was induced with 0.1 mM (final concentration) isopropyl β -D-1-thiogalactopyranoside (IPTG). After 16 h of induction at 303 K, the cells were harvested by centrifugation (7700g for 10 min at 277 K), resuspended in lysis buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 10 mM imidazole, 3 mM MgSO₄, 1.5 mM PMSF, 0.1 mg ml⁻¹ lysozyme, 20 μ g ml⁻¹ DNase) and lysed using a cell disruptor (83 MPa; Stansted). The supernatant was collected by centrifugation (35 000g for 45 min at 277 K) and applied onto a HiTrap Chelating HP column (GE Healthcare) equilibrated with buffer A (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 5% glycerol, 2 mM MnCl₂) plus 10 mM imidazole. Nonspecifically bound proteins were washed out and AtMnSOD was eluted with buffer A plus 500 mM imidazole. AtMnSOD-containing fractions were pooled, concentrated and loaded onto a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated with protein storage buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM DTT, 2 mM MnCl₂). Fractions containing AtMnSOD were pooled and concentrated to 25 mg ml⁻¹. The final protein solution showed a pink colour owing to the presence

of manganese, as confirmed by UV-Vis spectroscopy with a peak at 480 nm. The percentage of incorporated metal (~72%) was then quantified by the inductively coupled plasma atomic emission spectroscopy (ICP-AES) technique. Purified AtMnSOD appeared as a major band at ~25 kDa on SDS-PAGE gel analysis (Fig. 1a) and displayed SOD activity, as evaluated on a 10% polyacrylamide gel (Fig. 1b) using the NBT-staining method (Beauchamp & Fridovich, 1971).

2.2. Protein crystallization

Crystallization trials for AtMnSOD were set up as nanolitre-scale sitting drops using a Cartesian crystallization robot dispensing system (Genomics Solutions) and the PEG/Ion HT screen (Hampton Research). Conditions yielding the most promising crystals were further optimized using the hanging-drop vapour-diffusion method (1 μ l protein solution and 1 μ l reservoir solution) by varying the protein concentration, pH, precipitants and temperature. The best-diffracting crystals were obtained by mixing 1 μ l protein solution (15 mg ml⁻¹) with 1 μ l reservoir solution containing 0.2 M sodium formate, 20% PEG 3350 and appeared after 1 d at 293 K (Fig. 2a) and after one week at 277 K (Fig. 2b). Prior to data collection, the crystals were cryoprotected in reservoir solution supplemented with 20%(v/v) glycerol and flash-cooled in liquid nitrogen.

Figure 1
(a) SDS-PAGE analysis of purified AtMnSOD. Lane M contains molecular-weight marker (labelled in kDa). (b) Native PAGE gel stained for SOD activity with NBT. The white gel band owing to superoxide scavenging corresponds to the purified AtMnSOD, indicating that the protein is active.

(a)

(b)

Figure 2
AtMnSOD crystals grown in 0.2 M sodium formate, 20% PEG 3350 at 293 K (a) and 277 K (b).

Table 1

Diffraction data-collection and processing parameters.

Values in parentheses are for the highest resolution shell.

Beamline	ID29, ESRF
Wavelength (Å)	0.96112
Detector	PILATUS 6M
Oscillation angle per frame (°)	0.1
Total rotation range (°)	180
Space group	<i>P</i> 1
Unit-cell parameters (Å, °)	<i>a</i> = 58.95, <i>b</i> = 59.18, <i>c</i> = 107.98, $\alpha = 90.55$, $\beta = 90.51$, $\gamma = 89.85$
Mosaicity (°)	0.1
Resolution range (Å)	53.99–1.95 (2.07–1.95)
Total No. of reflections	171680 (22956)
No. of unique reflections	98683 (15861)
Completeness (%)	92.9 (92.3)
$ E^2 - 1 $	0.733
$\langle I/\sigma(I) \rangle^\dagger$	7.64 (1.09)
$R_{\text{merge}}^\ddagger$ (%)	6.6 (56.9)
$R_{\text{meas}}^\S$ (%)	9.3 (80.4)
$CC_{1/2}^\P$ (%)	99.7 (52.9)
Multiplicity	1.7 (1.4)
Overall <i>B</i> factor from Wilson plot (Å ²)	35
No. of molecules per asymmetric unit	8
Solvent content [%(<i>v/v</i>)]	41
<i>V_M</i> (Å ³ Da ^{−1})	2.072

[†] Diffraction images were processed up to the detector border and led to significant data in the highest resolution shell, according to the authors of the processing program (Karplus & Diederichs, 2012). $\langle I/\sigma(I) \rangle$ falls below 2.0 at 2.1 Å resolution. [‡] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity of multiple observations of symmetry-related reflections (Arndt *et al.*, 1968). [§] $R_{\text{meas}} = \sum_{hkl} [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $N(hkl)$ is the data multiplicity, $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity of multiple observations of symmetry-related reflections. It is an indicator of the average spread of the individual measurements (Diederichs & Karplus, 1997). [¶] $CC_{1/2}$ is the Pearson correlation coefficient between independently merged halves of the data set, as defined in Karplus & Diederichs (2012).

2.3. Data collection, processing and molecular replacement

Diffraction experiments on AtMnSOD crystals were performed on beamlines ID29 and ID23 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Diffraction images from various crystals were collected under a nitrogen stream at 100 K using a PILATUS 6M detector (Dectris). The data set presented here was obtained from 1800 images of consecutive 0.1° crystal oscillations, which were processed with the *XDS* program package (Kabsch, 2010). Self-rotation function calculations were performed with the program *POLARRFN* from the *CCP4* suite (Winn *et al.*, 2011) using normalized structure factors and a Patterson radius of 20 Å for data to 2.5 Å resolution. The normalized structure factors were produced with the program *ECALC* from the *CCP4* suite (Winn *et al.*, 2011). Molecular replacement was performed with *Phaser* (McCoy *et al.*, 2007) from the *PHENIX* package (Adams *et al.*, 2010).

3. Results and discussion

AtMnSOD crystals suitable for X-ray experiments were obtained by the hanging-drop vapour-diffusion method in 0.2 *M* sodium formate, 20% PEG 3350. Most crystals grown at 293 K (Fig. 2*a*) diffracted to only 3–5 Å resolution and their crystal parameters showed a seemingly tetragonal metric. Therefore, the strategy program at the ESRF stations proposed an automatic data-collection protocol using about 90° of crystal rotation, and an initial data set was collected accordingly. However, when the data set was tentatively reprocessed in tetragonal, orthorhombic, monoclinic and triclinic systems, R_{meas} values of 0.44–0.50, 0.41, 0.26–0.33 and 0.13 were obtained, respectively. Thus, the crystal in fact belonged to the triclinic space group *P*1, although strong NCS features and/or pseudo-merohedric twinning defects might also be present. Owing to the assumption of a

putative higher symmetry for data collection, the data set was far from being complete. Therefore, in a subsequent data collection a total crystal rotation of 180° was imposed, which allowed the collection of a fairly complete data set to 1.95 Å resolution from a crystal grown at 277 K (Fig. 2*b*). Data processing confirmed *P*1 as the crystal space group (Table 1).

Analysis of the crystal solvent content using the Matthews coefficient (Matthews, 1968) suggested the presence of seven or eight molecules in the asymmetric unit. Self-rotation function analysis revealed the presence of very strong noncrystallographic twofold rotation axes (Fig. 3*a*) parallel to the *a* and *b* directions and their diagonal. Such a set of twofold NCS rotation axes is in agreement

Figure 3 Self-rotation function plots for the *P*1 AtMnSOD crystal, showing contours ranging from 0 to 10 (origin peak). (a) $\kappa = 180^\circ$ section showing intense peaks at $\omega \approx 90^\circ$ approximately parallel to *a*, *b* or their diagonal. (b) $\kappa = 90^\circ$ section showing a single peak at $\omega \approx 0^\circ$.

with a fourfold NCS rotation axis (Fig. 3*b*) that is parallel to the *c* direction. The intensities of the peaks are remarkably high (up to 75% of the origin peak), which might result from possible twinning defects, but the $|E^2 - 1|$ statistics (Table 1) below 0.736 suggest otherwise. The set of NCS rotations suggests an asymmetric unit (or unit cell) with pseudo-422 point-group symmetry, and is compatible with the presence of eight monomers in the asymmetric unit. These results are compatible with the oligomerization state of prokaryotic MnSODs (which are generally dimeric) and eukaryotic MnSODs (which are generally tetrameric).

Structure determination by molecular replacement (MR) was carried out using an ensemble of SOD structures [PDB entries 3ak2 (Nakamura *et al.*, 2011), 1gv3 (Atzenhofer *et al.*, 2002), 1ix9 (B. F. Anderson, R. A. Edwards, M. M. Whittaker, J. W. Whittaker, E. N. Baker & G. B. Jameson, unpublished work), 1luw (Hearn *et al.*, 2003) and 1coj (Lim *et al.*, 1997); sequence identities below 55%], in which the insertions and flexible loops were removed, as a search model. An MR solution composed of eight SOD monomers was readily obtained by *Phaser*, and its final translation-function *Z*-score TFZ of 15.8 supported the correctness of the solution (Oeffner *et al.*, 2013). Detailed structural and kinetic analysis of AtMnSOD is in progress. Comparison of the structural and kinetic information obtained for AtMnSOD with that of other MnSODs will shed more light on the mechanism of this essential enzyme.

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References

- Abreu, I. A. & Cabelli, D. E. (2010). *Biochim. Biophys. Acta*, **1804**, 263–274.
- Abreu, I. A., Farinha, A. P., Negrão, S., Gonçalves, N., Fonseca, C., Rodrigues, M., Batista, R., Saibo, N. J. & Oliveira, M. M. (2013). *J. Proteomics*, **93**, 145–168.
- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Arndt, U. W., Crowther, R. A. & Mallett, J. F. (1968). *J. Sci. Instrum.* **1**, 510–516.
- Atzenhofer, W., Regelsberger, G., Jacob, U., Peschek, G., Furtmüller, P., Huber, R. & Obinger, C. (2002). *J. Mol. Biol.* **321**, 479–489.
- Beauchamp, C. & Fridovich, I. (1971). *Anal. Biochem.* **44**, 276–287.
- Diederichs, K. & Karplus, P. A. (1997). *Nature Struct. Biol.* **4**, 269–275.
- Fridovich, I. (1995). *Annu. Rev. Biochem.* **64**, 97–112.
- Gill, S. S. & Tuteja, N. (2010). *Plant Physiol. Biochem.* **48**, 909–930.
- Hearn, A. S., Stroupe, M. E., Cabelli, D. E., Ramilo, C. A., Luba, J. P., Tainer, J. A., Nick, H. S. & Silverman, D. N. (2003). *Biochemistry*, **42**, 2781–2789.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Karplus, P. A. & Diederichs, K. (2012). *Science*, **336**, 1030–1033.
- Lim, J. H., Yu, Y. G., Han, Y. S., Cho, S., Ahn, B.-Y., Kim, S.-H. & Cho, Y. (1997). *J. Mol. Biol.* **270**, 259–274.
- Martin, M. V., Fiol, D. F., Sundaresan, V., Zabaleta, E. J. & Pagnussat, G. C. (2013). *Plant Cell*, **25**, 1573–1591.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Miller, A. F. (2012). *FEBS Lett.* **586**, 585–595.
- Morgan, M. J., Lehmann, M., Schwarzländer, M., Baxter, C. J., Sienkiewicz-Porzućek, A., Williams, T. C., Schauer, N., Fernie, A. R., Fricker, M. D., Ratcliffe, R. G., Sweetlove, L. J. & Finkemeier, I. (2008). *Plant Physiol.* **147**, 101–114.
- Nakamura, T., Torikai, K., Uegaki, K., Morita, J., Machida, K., Suzuki, A. & Kawata, Y. (2011). *FEBS J.* **278**, 598–609.
- Oeffner, R. D., Bunkóczi, G., McCoy, A. J. & Read, R. J. (2013). *Acta Cryst.* **D69**, 2209–2215.
- Sheng, Y., Stich, T. A., Barnese, K., Gralla, E. B., Cascio, D., Britt, R. D., Cabelli, D. E. & Valentine, J. S. (2011). *J. Am. Chem. Soc.* **133**, 20878–20889.
- Wang, F.-Z., Wang, Q.-B., Kwon, S.-Y., Kwak, S.-S. & Su, W.-A. (2005). *J. Plant Physiol.* **162**, 465–472.
- Wang, Y., Wisniewski, M., Meilan, R., Uratsu, S. L., Cui, M., Dandekar, A. & Fuchigami, L. (2007). *J. Appl. Hortic.* **9**, 3–8.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Zelko, I. N., Mariani, T. J. & Folz, R. J. (2002). *Free Radic. Biol. Med.* **33**, 337–349.