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GPX3 from *Arabidopsis thaliana*: cloning, expression, purification, crystallization and preliminary X-ray analysis

The *Arabidopsis thaliana* glutathione peroxidase 3 (GPX3) gene encodes a glutathione peroxidase with roles in H₂O₂ homeostasis and signalling. The GPX3 gene sequence was cloned into pGEX-6P1 and overexpressed in *Escherichia coli*. The GPX3 protein was purified to homogeneity in two chromatographic steps. Various lengths of the GPX3 sequence were used to obtain proteins that yielded crystals using vapour-diffusion techniques, but only GPX3ΔN36 (lacking 36 amino acids from the N-terminus) showed a good diffraction pattern. Its crystals diffracted to 2.8 Å resolution and belonged to space group P6₅, with unit-cell parameters $a = b = 98.241$, $c = 42.057$ Å.

1. Introduction

In aerobic organisms, reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide are generated during the incomplete reduction of molecular oxygen to water (Farr & Kogoma, 1991), after exposure to environmental factors (Dat *et al.*, 2000) and/or during various metabolic processes (*e.g.* photosynthesis and respiration; Milla *et al.*, 2003). Plants have evolved different enzymatic and non-enzymatic mechanisms to cope with increased levels of ROS (Apel & Hirt, 2004; Mittler *et al.*, 2004). The major non-enzymatic antioxidant compounds are carotenoids, tocopherols, reduced glutathione (GSH) and ascorbate (Noctor & Foyer, 1998). The components of enzymatic systems include superoxide dismutases, catalases (CATs), ascorbate peroxidases (APXs), peroxiredoxins (PRXs) and glutathione peroxidases (GPXs).

GPXs are a group of enzymes that regulate the levels of ROS in cells and tissues, protecting them against oxidative damage. In mammals, a selenocysteine residue in GPXs plays an important role in catalysis. When the selenocysteine is exchanged for cysteine by site-directed mutagenesis, the catalytic activity of the GPX decreases by two to three orders of magnitude (Rocher *et al.*, 1992; Maiorino *et al.*, 1995). Typical plant GPXs have sequences similar to those of animal GPXs, but most higher plant GPX homologues studied so far have a cysteine residue instead of a selenocysteine residue in their active site (Stadtman, 1996). Consequently, plant GPXs have lower activities than animal GPXs because they contain Cys at the putative catalytic site instead of selenocysteine (Holland *et al.*, 1993; Faltin *et al.*, 1998; Herbette *et al.*, 2002; Jung *et al.*, 2002). In GPXs containing selenocysteine (Sec-GPXs), the reaction takes place at a single redox centre with selenocysteine as the redox-active residue. Their redox centre comprises (seleno)cysteine, tryptophan, asparagine and glutamine (Toppo *et al.*, 2009). However, less is known about the redox centre of cysteine-containing GPXs.

ATGPX3 is one of eight GPXs in the ATGPX family in *Arabidopsis*. It plays a dual role in plants: it controls H₂O₂ homeostasis and relays an H₂O₂ signal in guard cells. This signal mediates stomatal regulation in response to abscisic acid (ABA) and drought stress *via* activating calcium channels and the expression of ABA-responsive and stress-responsive genes. ATGPX3 physically interacts with the 2C-type protein phosphatase ABI2 and to a lesser extent with ABI1. In addition, the redox states of both ATGPX3 and ABI2 are regulated by H₂O₂ (Miao *et al.*, 2006).

Structural characterization of GPX3 is central to understanding the regulatory mechanisms of plant GPXs. In this work, we describe

the expression, purification, crystallization and preliminary crystallographic analysis of the transmembrane protein GPX3. The expression, purification and crystallization of eukaryotic proteins using bacterial expression systems is a bottleneck in many structural studies, especially those of components of signal transduction cascades. We overcame these problems by reducing the concentration of the inducer isopropyl β -D-1-thiogalactopyranoside to 0.01 mM, by using Rosetta (Novagen), a bacterial strain that avoids codon-bias problems, and by creating a GST-fusion protein with improved protein solubility (Frangioni & Neel, 1993). To prevent the transmembrane region affecting the solubility and purification of the protein, we expressed the GPX3 protein without the final 36 amino acids at the N-terminus; this region is the predicted transmembrane region and inside region (as predicted using *TMHMM* v.2.0; Krogh *et al.*, 2001).

2. Materials and methods

2.1. GPX3 gene cloning

The GPX3 gene from *A. thaliana* encodes a protein with an outside membrane region comprising 170 amino acids. The sequence corresponding to the outside membrane region was named GPX3 Δ N36 because it lacked 36 amino acids from the N-terminus. GPX3 Δ N36 was cloned into the expression plasmid pGEX-6P1 (GE Healthcare, Beijing, People's Republic of China) between the *Bam*HI and *Xho*I sites. The primers used were as follows: forward, 5'-GAT GGA TCC

TCG CCA TCG ACG GTG GAA CAA-3'; reverse, 5'-CAC CTC GAG TCA AGC AGA TGC CAA TAG CTT-3'. This construct encoded a GST-fusion protein with eight amino acids (LEVLFQGP) between the PreScission protease cleavage site and the start of GPX3.

2.2. Protein expression and purification

The pGEX-6P1-GPX3 Δ N36 plasmid was transformed into *Escherichia coli* strain Rosetta (DE3) for protein expression using standard protocols (Sambrook & Russell, 2001). A 5 ml aliquot of an overnight culture was subcultured into 800 ml fresh Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract and 10 g NaCl per litre of solution) containing ampicillin (50 mg l⁻¹). Transformed cells were grown at 310 K. When the optical density (OD) at 600 nm reached 0.5–0.8, the cells were incubated at 277 K for 5 min. Protein expression was induced by shaking cells (200 rev min⁻¹ in a rocking incubator) with 0.3 mM isopropyl β -D-1-thiogalactopyranoside overnight at 289 K. The cells were harvested by centrifugation (15 min, 3500g), resuspended in PBS buffer (0.24 g l⁻¹ KH₂PO₄, 1.44 g l⁻¹ Na₂HPO₄, 8.0 g l⁻¹ NaCl, 0.2 g l⁻¹ KCl, pH 7.3) and disrupted by sonication.

After centrifugation for 40 min at 23 000g and 277 K, the clear supernatant was filtered through a 0.45 μ m pore-size filter (Millipore, Bedford, Massachusetts, USA). The GST-tagged GPX3 was purified using Glutathione Sepharose beads (GE Healthcare) according to the manufacturer's instructions. The filtered supernatant was mixed with the previously equilibrated beads. After incubation, a washing step was performed with ten volumes of PBS buffer. SDS-PAGE analysis revealed that the molecular mass of GST-GPX3 Δ N36 was 44.9 kDa. The GPX3 protein was cleaved from GST using PreScission protease, which was purified in our laboratory (~2 mg ml⁻¹). The cleavage step yielded protein fragments corresponding to the GST protein (26 kDa) and to GPX3 Δ N36 (18.9 kDa). During the chromatography and enzymatic digestion steps, the protein mixtures were monitored by SDS-PAGE (Fig. 1*a*). A final polishing step was performed using Superdex 75 16/60 (Amersham Biosciences; Fig. 1*b*). GPX3 was concentrated to a final concentration of 15 mg ml⁻¹ using a 10 kDa cutoff Amicon protein concentrator (YM-10; Millipore) in 20 mM Tris-HCl pH 8.0, 150 mM NaCl. The final protein concentration was determined spectrophotometrically using the calculated molar absorption coefficient at 280 nm (30 370 M⁻¹ cm⁻¹; Gasteiger *et al.*, 2005). Samples were kept at 277 K until analysis.

2.3. Crystallization

Crystallization conditions for GPX3 Δ N36 were screened using the commercial screens Crystal Screen, Crystal Screen 2, PEG/Ion and Index (Hampton Research, Laguna Niguel, California, USA). Crystallization assays were carried out using the hanging-drop vapour-diffusion method at 289 K in 16-well plates with two concentrations of GPX3. For crystallization, every drop consisted of 1 μ l protein solution at 7.5 or 15 mg ml⁻¹ in buffer (20 mM Tris-HCl, 150 mM NaCl pH 8.0) and 1 μ l precipitant solution, which were mixed and equilibrated in the 16-well plates.

The crystals of GPX3 Δ N36 used in our analyses were obtained using condition No. 23 of Crystal Screen 2 [10%(v/v) 1,4-dioxane, 1.6 M ammonium sulfate, 0.1 M MES monohydrate pH 6.5].

2.4. Data collection and analysis

Crystals were mounted in a fibre loop, transferred into cryo-protectant solution (20% glycerol) and flash-cooled at 100 K in a nitrogen-gas stream. Preliminary diffraction data were collected on an in-house MAR 165 CCD detector (MAR Research, Hamburg,

Figure 1
(*a*) SDS-PAGE analysis of purified GPX3 Δ N36. Lane 1, molecular-mass marker (labelled in kDa). Lane 2 corresponds to the products after PreScission protease cleavage. Lane 3 corresponds to the cleavage product obtained from GST-fused GPX3 Δ N36 after elution with 10 mM GSH buffer. (*b*) Size-exclusion chromatogram of GPX3 Δ N36. The plot shows absorbance at 280 nm.

Germany) at the Laboratory of Structural Biology, School of Medicine, Tsinghua University, Beijing, People's Republic of China. The GPX3ΔN36 crystals showed good-quality diffraction patterns (Fig. 2). All intensity data were indexed, integrated and scaled with the *HKL-2000* package (Otwinowski & Minor, 1997). A complete data set was collected to 2.8 Å resolution using the in-house X-ray source. A summary of the diffraction protocol and data-collection statistics is given in Table 1.

3. Results and discussion

GPX3 is a membrane protein. It is located in the Golgi apparatus, cytoplasm, endosome, mitochondrion and trans-Golgi network (<http://www.arabidopsis.org/>). To obtain soluble protein, GPX3ΔN36 (lacking 36 amino acids at the N-terminus), GPX3ΔN87 (lacking 87 amino acids at the N-terminus) and GPX3 were expressed as GST-fusion proteins. However, only GPX3ΔN36 yielded sufficient soluble protein for crystallization analyses (data not shown). The purity of GPX3ΔN36 at the final purification step was at least 95% as monitored by SDS-PAGE (Fig. 1a). In Fig. 1, lane 3 corresponds to the product after PreScission protease cleavage. Size-exclusion chromatography suggested that GPX3ΔN36 was monomeric (Fig. 1b).

GPX3ΔN36 was purified in PBS buffer, exchanged into PBS buffer (20 mM Tris-HCl, 150 mM NaCl pH 8.0) using a 10 kDa cutoff Amicon protein concentrator and further purified on Superdex 75 using an ÄKTA FPLC purifier. In general, the crystals of GPX3ΔN36 appeared in 9–10 d and continued to grow to maximum dimensions (90 µm) over the following week. Data were collected using 20% glycerol as the cryoprotectant.

The GPX3ΔN36 crystals displayed a good-quality diffraction pattern (Fig. 2). The crystals diffracted to 2.8 Å resolution and belonged to space group *P6₅*, with unit-cell parameters $a = b = 98.241$, $c = 42.057$ Å. The strategy used to solve the GPX3ΔN36 structure was molecular replacement with the program *MOLREP* (Vagin & Teplyakov, 2010) using the coordinates of *Populus trichocarpa* GPX5

Table 1

Diffraction protocol and data-collection statistics for GPX3ΔN36 crystals.

Values in parentheses are for the outermost resolution shell.

Unit-cell parameters	
a (Å)	98.241
b (Å)	98.241
c (Å)	42.057
α (°)	90.00
β (°)	90.00
γ (°)	120.00
Space group	<i>P6₅</i>
Wavelength (Å)	1.000
Resolution (Å)	25.55–2.02
Total No. of reflections	180059 (8404)
No. of unique reflections	15347 (764)
Completeness (%)	95.94
Average $I/\sigma(I)$	18.5 (8.2)
$R_{\text{merge}}^{\dagger}$	0.101 (0.476)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean of the observations $I_i(hkl)$ of reflection hkl .

(PDB entry 2p5q; Koh *et al.*, 2007) as the model. *P. trichocarpa* GPX5 shows 67% sequence identity to *A. thaliana* GPX3.

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Figure 2

X-ray diffraction pattern of a GPX3ΔN36 crystal obtained using a synchrotron-radiation source. The inset shows a magnification of a sector of the diffraction frame. The numbers give the resolutions of the circles (in Å).