Crystallization of the C-terminal redox domain of the sulfur-assimilatory enzyme APR1 from *Arabidopsis thaliana*

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Received 25 April 2014

Accepted 5 July 2014

Plant-type APS reductase (APR), which catalyzes the reduction of activated sulfate to sulfite in plants, consists of a reductase domain and a C-terminal redox domain showing sequence homology to thioredoxin but possessing the activity of glutaredoxin. In order to understand the structural and biochemical properties of the redox domain of plant-type APS reductase, the C-terminal domain of APR1 (APR1C) from *Arabidopsis thaliana* was crystallized using the sitting-drop vapour-diffusion method. X-ray diffraction data were collected to a resolution of 2.70 Å on the SPXF beamline BL13B1 at the NSRRC, Taiwan. The crystals belonged to space group $P4_32_12$ or $P4_12_12$, with unit-cell parameters $a = b = 58.2$, $c = 86.7$ Å. With one molecule per asymmetric unit, the crystal volume per unit protein weight (V_M) is $2.64 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of approximately 53.49%. Further structure-based functional studies of APR1C would extend knowledge of the molecular mechanism and regulation of APR.

1. Introduction

Sulfur is an essential element that plays significant roles in many metabolic processes. It is present in the amino acids cysteine and methionine, and thus is an important component of peptides and proteins. Many enzymes require sulfur-containing coenzymes and prosthetic groups for their activity. Other cofactors, proteins and lipids containing sulfur, and sulfonate groups modifying polysaccharides, are also physiological essentials for all organisms (Beinert, 2000; Chen *et al.*, 2011). Additionally, a variety of sulfur-containing secondary metabolites are produced in plants, which often play an important role in defence against pathogens and herbivores (Davidian & Kopriva, 2010; Pinto *et al.*, 2007). For incorporation into bio-organic compounds, oxidized inorganic sulfur compounds (usually sulfate) have to be reduced in the assimilatory sulfate-reduction pathway.

Plant-type adenosine 5'-phosphosulfate reductase (APR) is necessary for plant growth (Loudet *et al.*, 2007) and tolerance to heavy metals (Grant *et al.*, 2011), and directly connects the sulfur-assimilation pathway to the biosynthesis of sulfonated molecules (Loudet *et al.*, 2007; Hermsen *et al.*, 2010; Lee *et al.*, 2011; McCallum *et al.*, 2011; Thomas *et al.*, 2011). The mature APR consists of two distinct domains, a sulfonucleotide reductase-like domain and a thioredoxin-like domain. A truncated plant-type APR lacking the C-terminal redox domain shows low APR activity, but this function could be recovered after the addition of thioredoxin (Prior *et al.*, 1999; Bick *et al.*, 1998). In addition, recombinant thioredoxin m enhanced the reaction velocity but not thioredoxin f (Weber *et al.*, 2000). The APR reaction can thus be divided into three independent steps: transfer of sulfate from APS (adenosine 5'-phosphosulfate) to the active cysteine residue, release of the sulfite by the C-terminal redox domain and recovery of the active enzyme by reaction with thiols. Although the sequence of the C-terminal domain of APR is

Table 1

Macromolecule-production information.

Source organism	<i>A. thaliana</i>
DNA source	At4G04610.1
Forward primer	AAT TGA ATT CAT GGA GAA TCT TGT GAC TTT G
Reverse primer	AAT TCT CGA GCA AGA ACG AAG TCA AAG ACT
Cloning vector	pET-28a
Expression vector	pGEX-4T1
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	GSPEFMENLVTLRSQGIEIENLMKLENRKEPWIVVLYAPWCPFC-QAMEASYDELADKLAGSGIKVAKFRADGDQKEFAKQELQL-GSFPTILVFPKNSSRPKIKYPSEKRDVESLTSFL

more similar to thioredoxin than to glutaredoxin, it possesses the enzymatic activity of glutaredoxin (Bick *et al.*, 1998; Bick & Leustek, 1998). Glutaredoxin does not require an enzymatic reduction, like that of thioredoxin by thioredoxin reductase, but is reduced by glutathione (GSH). The formation of free sulfite by APR from *Lemna minor* using GSH at physiological concentrations as the reductant has been demonstrated (Suter *et al.*, 2000).

Evidence has shown that the catalytic mechanism of plant-type APS reductase depends on the interaction of two domains, but the controversy about the regulatory role of the C-terminal redox domain of this enzyme still needs to be resolved. For further understanding of the structural and functional relationship, we have initiated the crystal structure solution of the redox domain of APR1 from *Arabidopsis thaliana* (APR1C). Here, the cloning, expression, purification, crystallization and X-ray analysis of APR1C are reported.

2. Materials and methods

2.1. Macromolecule production

2.1.1. Molecular cloning. The DNA encoding amino acids 353–461 of *A. thaliana* APR1 (465 amino acids, TAIR accession No. At4G04610.1) was amplified by PCR using a prepared cDNA library and the gene-specific forward and reverse primers listed in Table 1. The PCR product was cloned into the plasmid pGEX-4T1 (GE

Table 2

Crystallization.

Method	Sitting drop
Plate type	Cryschem plate
Temperature (K)	283
Protein concentration (mg ml ⁻¹)	3.5
Buffer composition of protein solution	20 mM Tris-HCl pH 7.0, 200 mM NaCl
Composition of reservoir solution	0.1 M Tris buffer pH 7.0, 1.2 M sodium citrate, 0.2 M sodium chloride
Volume and ratio of drop	2 µl (1:1 ratio)
Volume of reservoir (µl)	400

Healthcare) that encodes an upstream glutathione *S*-transferase (GST) tag followed by a thrombin cleavage site. *Escherichia coli* strain DH5α (Novagen) was used for plasmid cloning, and the insertion of the gene for the APR1 glutaredoxin domain was confirmed by DNA sequencing.

2.1.2. Protein expression. The recombinant plasmid was transformed into *E. coli* strain BL21(DE3) (Novagen) for protein expression. The transformed cells were cultured at 310 K in Luria-Bertani broth containing 50 mg l⁻¹ ampicillin. When the optical absorption density at 600 nm reached 0.6, protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was incubated for a further 17 h at 298 K. The cells were then harvested by centrifugation at 9000g for 30 min at 277 K.

2.1.3. Protein purification. The cell pellets were resuspended in lysis buffer consisting of 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl pH 7.4 and disrupted by ultrasonication. Cell debris was removed by centrifugation at 20 000g for 30 min at 277 K. The resulting supernatant was loaded onto a Glutathione-Sepharose 4B affinity column (GE Healthcare) pre-equilibrated with the lysis buffer. After extensive washing with PBS buffer, GST-tagged APR1C was eluted from the column using elution buffer consisting of 50 mM Tris-HCl pH 8.0, 10 mM glutathione. SDS-PAGE analysis revealed that the molecular mass of GST-APR1C was 39 kDa. The APR1C protein with an extra Gly-Ser-Pro-Glu-Phe-Met sequence at the N-terminus was cleaved from GST by incubation with thrombin protease overnight at 283 K. The cleavage step yielded protein fragments corresponding to the GST protein (26 kDa) and to APR1C (13 kDa). During the chromatography and enzymatic digestion steps, the protein mixtures were monitored by SDS-PAGE (Fig. 1). After gel filtration using a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare) and elution with buffer (20 mM Tris-HCl pH 7.0, 200 mM NaCl), the protein was concentrated to 3.5 mg ml⁻¹ using an ultrafiltration system (Vivaspin, 3 kDa cutoff). Protein concentration was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976). The purity of APR1C was determined by Coomassie-Blue-stained SDS-PAGE analysis and was found to be 98% (Fig. 1).

2.2. Crystallization

Initial crystallization screening was performed at 283 K by the sitting-drop vapour-diffusion method using commercial crystallization screen kits, 96-well Intelli-Plates (Hampton Research) and a HoneyBee 963 robot (Genomic Solutions) according to a previously described protocol (Chang *et al.*, 2013). Each crystallization drop was prepared by mixing 0.3 µl reservoir solution and 0.3 µl protein solution, and the mixture was equilibrated against 0.1 ml reservoir solution. After four weeks, crystals appeared in 0.1 M Tris buffer pH 7.0, 1 M sodium citrate, 0.2 M sodium chloride. Conditions were further optimized by varying the pH value and precipitant concentrations to

Figure 1

15% SDS-PAGE analysis of purified APR1C stained with Coomassie Brilliant Blue. Lane 1, protein markers (labelled in kDa). Lane 2 corresponds to the product after thrombin protease cleavage. Lane 3 corresponds to the cleavage product obtained from GST-fused APR1C after elution with 10 mM GSH buffer. Lane 4 corresponds to the final concentrated APR1C showing high purity.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL13B1, NSRRC, Taiwan
Wavelength (Å)	1.0
Temperature (K)	100 K
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	30
Space group	$P4_32_12$ or $P4_12_12$
a, b, c (Å)	58.2, 58.2, 86.7
α, β, γ (°)	90, 90, 90
Mosaicity (°)	0.351
Resolution range (Å)	50.00–2.70 (2.80–2.70)
Total No. of reflections	57471 (5450)
No. of unique reflections	4467 (432)
Completeness (%)	99.6 (100.0)
Multiplicity	12.9 (12.3)
$\langle I/\sigma(I) \rangle$	32.4 (4.7)
$R_{\text{merge}}^\dagger$	0.053 (0.482)
$R_{\text{r.i.m.}}^\ddagger$	0.079 (0.659)
Overall B factor from Wilson plot (Å ²)	61.11

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ are the intensities of the individual replicates of a given reflection hkl and $\langle I(hkl) \rangle$ is the average intensity over all replicates of that reflection. ‡ Estimated $R_{\text{r.i.m.}} = R_{\text{merge}}[N/(N-1)]^{1/2}$, where N is the data multiplicity.

obtain diffraction-quality crystals (Table 2). The crystals for data collection grew in two weeks at 283 K with the optimal condition consisting of 0.1 M Tris buffer pH 7.0, 1.2 M sodium citrate, 0.2 M sodium chloride. The approximate dimensions of the crystals were $30 \times 30 \times 30 \mu\text{m}$ (Fig. 2).

2.3. Data collection and processing

Data were collected from APR1C crystals at a wavelength of 1.0 Å at 100 K on the protein crystallographic SPXF beamline BL13B1 at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan equipped with an ADSC Q315r detector. Single crystals were transferred into the mother liquor containing 20% (v/v) glycerol as a cryoprotectant for 1 min, mounted in a 0.1 mm nylon loop (Hampton Research) and then flash-cooled in liquid nitrogen to 100 K. The oscillation angle was 1°. Diffraction images were indexed, integrated and scaled using *DENZO* (Rossmann & van Beek, 1999) and *SCALEPACK* (Borek *et al.*, 2003) from the *HKL-2000* program suite

Figure 2

Crystals of APR1C obtained in 0.1 M Tris buffer pH 7.0, 1.2 M sodium citrate, 0.2 M sodium chloride by the sitting-drop vapour-diffusion method. The approximate dimensions of the crystals are $30 \times 30 \times 30 \mu\text{m}$.

Figure 3

Typical diffraction image obtained from crystals of APR1C collected at a synchrotron-radiation source (beamline 13B1, NSRRC, Taiwan). Diffraction extends to 2.70 Å resolution.

(Otwinski & Minor, 1997). A complete data set was obtained to 2.70 Å with an R_{merge} of 5.3%. Details of the data-collection statistics are summarized in Table 3.

3. Results and discussion

The DNA encoding amino acids 353–461 of *A. thaliana* APR1 was cloned into a bacterial expression plasmid for protein production. The overexpressed protein was purified to homogeneity using a two-step procedure. Using the N-terminal GST tag of recombinant APR1C, we were able to purify the protein with a GST-affinity column. The GST tag was then excised from APR1C with thrombin protease at 283 K, resulting in a Gly-Ser-Pro-Glu-Phe-Met artificial sequence at the N-terminus of APR1C. Subsequent gel-filtration chromatography was used to isolate a homogeneous protein sample. The purity of the protein was analyzed by SDS-PAGE and it was found to be about 98% pure. Crystals were obtained using the sitting-drop vapour-diffusion technique at 283 K. On the basis of the diffraction pattern (Fig. 3), the APR1C crystal belonged to the tetragonal space group $P4_32_12$ or its enantiomer $P4_12_12$, with unit-cell parameters $a = b = 58.2$, $c = 86.7$ Å. Assuming the presence of one molecule of the 13 kDa protein in the asymmetric unit, the calculated Matthews coefficient (V_M) is $2.64 \text{ Å}^3 \text{ Da}^{-1}$, with a solvent content of 53.49% (Matthews, 1968). Further structural and biochemical analysis are currently under way.

This work was supported by the National Science Council (NSC102-2113-M-002-005 and NSC101-2113-M-002-017) and National Taiwan University (NTU-ERP-103R8600 and NTU-ICRP-103R7560-5) in Taiwan. We also thank the Technology Commons in College of Life Science and Center for Systems Biology, National Taiwan University for instrumental support of protein crystallization.

Portions of this research were carried out at the National Synchrotron Radiation Research Center, a national user facility supported by the National Science Council of Taiwan. The Synchrotron Radiation Protein Crystallography Facility is supported by the National Core Facility Program for Biotechnology.

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