Recombinant ACHT1 from *Arabidopsis thaliana*: crystallization and X-ray crystallographic analysis

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Thioredoxins (Trxs) play important roles in chloroplasts by linking photosynthetic light reactions to a series of plastid functions. They execute their function by regulating the oxidation and reduction of disulfide bonds. ACHT1 (atypical cysteine/histidine-rich Trx1) is a thylakoid-associated thioredoxin-type protein found in the *Arabidopsis thaliana* chloroplast. Recombinant ACHT1 protein was overexpressed in *Escherichia coli*, purified and crystallized by the vapour-diffusion method. The crystal diffracted to 1.7 Å resolution and a complete X-ray data set was collected. Preliminary crystallographic analysis suggested that the crystals belonged to space group $C22_1$, with unit-cell parameters $a = 102.7$, $b = 100.6$, $c = 92.8$ Å.

1. Introduction

Thioredoxins (Trxs) are ubiquitous disulfide reductases and are key actors in cellular redox regulation in almost all life-forms (Meyer *et al.*, 2009). Trxs were discovered about 50 years ago in bacteria, and since then their function and structure have been studied extensively. Trxs perform their functions by donating electrons to enzymes in numerous physiological processes, such as DNA synthesis, sulfur assimilation and regulation of transcription factors (Arnér & Holmgren, 2000). Whereas most organisms have small numbers of Trxs that perform multiple functions, plants have a great variety of Trxs (Meyer *et al.*, 2008; Geigenberger *et al.*, 2017). The study of *Arabidopsis thaliana* thioredoxins indicated that the number of genes encoding Trxs and Trx-like proteins was greater than 40 (Meyer *et al.*, 2005, 2006).

The *A. thaliana* atypical cysteine/histidine-rich Trxs (ACHTs) constitute a small family of plant-specific and chloroplast-localized Trxs (Dangoor *et al.*, 2009). ACHTs have an atypical redox-active site and a less reducing redox midpoint than those of canonical chloroplast Trxs, which differentiate them from classical Trxs and raise the possibility of a specialized redox function in plant chloroplasts. *In vitro* studies show that ACHTs catalyse the reduction of 2-Cys peroxiredoxin, but not of malate dehydrogenase (Dangoor *et al.*, 2009). A study of the thylakoid-associated ACHT1 indicates that it transmits a disulfide-based oxidative signal in feedback regulation of photosynthesis under homeostatic growth conditions (Dangoor *et al.*, 2012). Shortly after illuminating dark-adapted plants with moderate light intensity, the chloroplast-localized ACHT1 reduces 2-Cys peroxiredoxin and is itself oxidized (Dangoor *et al.*, 2012). The reduced 2-Cys peroxiredoxin then reduces hydrogen peroxide and organic peroxides produced during photosynthesis (Hall *et al.*, 2009; Dietz, 2011). The characteristics of the reaction of ACHT1 and 2-Cys peroxiredoxin in plants suggest that the oxidation of

Table 1
Protein-production information.

Source organism	<i>A. thaliana</i>
DNA source	cDNA
Forward primer	CGCGGATCCGCGGTCCAAGCGTTAGCTGCT
Reverse primer	GATCTCGAGTTATTCACCTTGAATCTT
Expression vector	pET-28a(+)
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced†	MGSSHHHHHSSGLVPRGSHMASMTGGQMGRGS-VQALAAETECPKWERKAGPNMIDITSAEQFLNALKDAGDRLVIVDFYGTWCGSCRAMFPKLCKTAKEHPNIFLKVNFENKSLCKSLNVKVLPHYFHYRGADGQVESFSCSLAKFKLREATERHN-VGSISNISSSASEKVEDSSE

† The His₆-tag sequence at the N-terminus of the recombinant ACHT1 protein is underlined.

ACHT1 is linked to changes in the photosynthetic production of peroxides.

The structures of prokaryotic and animal Trxs have been revealed (Holmgren *et al.*, 1975; Forman-Kay *et al.*, 1991). The structures of chloroplast Trxs from the green alga *Chlamydomonas reinhardtii* and from spinach have also been studied (Lancelin *et al.*, 2000; Capitani *et al.*, 2000). All of the above-mentioned Trxs with known structures are typical Trxs with a canonical C(G/P)PC motif at their redox site. Despite the wealth of recent studies revealing the function of the ACHTs, the structural basis and the mechanism of the oxidation of ACHTs by 2-Cys peroxiredoxin remain elusive. Here, we report the expression, purification, crystallization and preliminary crystallographic analysis of recombinant ACHT1 from *A. thaliana*.

2. Materials and methods

2.1. Macromolecule production

The ACHT1 cDNA fragment corresponding to residues 74–221 of AT4G26160 was amplified by PCR from an *A. thaliana* cDNA library with the forward primer 5'-CGCGGATCCGCGGTCCAAGCGTTAGCT-3' (the underlined bases correspond to the BamHI site) and the reverse primer 5'-GATCTCGAGTTATTCACCTTGAATCTT-3' (the underlined bases correspond to the XhoI site). The PCR product was cloned into the expression vector pET-28a(+) and then transformed into *Escherichia coli* BL21 (DE3) cells. The pET-28a(+)-ACHT1 expression plasmid was identified by restriction-endonuclease digestion and was further verified by DNA sequencing (Sangon Biotech, Shanghai, People's Republic of China). The expressed recombinant ACHT1 protein contains an N-terminal His₆ tag (Table 1). The cell culture was grown at 310 K in LB medium containing 30 µg ml⁻¹ kanamycin sulfate until the OD₆₀₀ reached 0.8. Expression of the recombinant protein was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside and the culture was incubated for an additional 18 h at 289 K. The cells were harvested by centrifugation at 5000g for 15 min at 277 K.

For the purification of recombinant ACHT1, the cells were resuspended in buffer A [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 5% (v/v) glycerol] and lysed by sonication.

The lysate was centrifuged at 10 000g for 1 h at 277 K and the supernatant was loaded onto an Ni-NTA column (Novagen) pre-equilibrated with buffer A. The column was eluted with buffer A supplemented with 100 mM imidazole. The fractions containing the eluted ACHT1 were pooled and concentrated before loading them onto a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated with buffer A. Fractions corresponding to ACHT1 were pooled, desalted by dialysis against 20 mM Tris-HCl pH 7.5, 30 mM NaCl, 1 mM DTT, 5% (v/v) glycerol and concentrated to 13.5 mg ml⁻¹ for crystallization.

The presence and purity of the recombinant ACHT1 were monitored by SDS-PAGE. The protein concentration was determined by a Bio-Rad protein assay with bovine serum albumin as a standard (Bradford, 1976).

2.2. Crystallization

Crystallization trials were carried out at 277 K by the sitting-drop vapour-diffusion method and the initial trials were performed using Crystal Screen and Crystal Screen 2 from Hampton Research. The 2 µl sitting drops consisted of 1 µl protein solution and 1 µl reservoir solution and were equilibrated against 100 µl reservoir solution. To improve the crystal quality, optimization of the crystallization condition was performed using the hanging-drop vapour-diffusion method in 24-well plates with 4 µl sitting drops consisting of 2 µl protein solution and 2 µl reservoir solution.

2.3. Data collection and processing

Diffraction data were collected on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF) at 100 K. Crystals were loop-mounted and flash-cooled in liquid nitrogen. The diffraction data were indexed, integrated and scaled using HKL-2000 (Otwinowski & Minor, 1997).

Figure 1
Size-exclusion chromatography and SDS-PAGE (inset) of ACHT1. In the SDS-PAGE, lane M contains molecular-weight markers (labelled in kDa). The other lanes are labelled with the corresponding elution volumes in ml.

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion for initial screening, hanging-drop vapour diffusion for crystal optimization
Plate type	48-well plates for initial crystal screening, 24-well plates for crystal optimization
Temperature (K)	277
Protein concentration (mg ml ⁻¹)	13.5
Buffer composition of protein solution	20 mM Tris-HCl pH 7.5, 30 mM NaCl, 1 mM DTT, 5%(w/v) glycerol
Composition of reservoir solution	2.0 M ammonium sulfate, 8%(v/v) 2-propanol, 0.005%(w/v) agarose
Volume and ratio of drop	2 µl, 1:1 ratio of protein:reservoir solution for initial crystal screening; 4 µl, 1:1 ratio of protein:reservoir solution for crystal optimization
Volume of reservoir	100 µl for initial screening, 200 µl for crystal optimization

3. Results and discussion

The full-length cDNA of ACHT1 contains 666 nucleotides and codes for a protein of 221 amino acids. Amino acids 1–73 are a transit peptide according to analysis in UniProt (<http://www.uniprot.org>). Therefore, the nucleotides encoding amino acids 74–221 were constructed in a pET-28a(+) expression plasmid and the recombinant protein was overexpressed in *E. coli* BL21 (DE3) cells. Recombinant ACHT1 with an N-terminal His₆ tag was successfully produced. Soluble protein was obtained after sonication and was eluted from an Ni-NTA affinity column. The target protein was eluted with 100 mM imidazole and was further purified by gel filtration. The collected fractions show a single protein band of approximately 16 kDa on SDS-PAGE, which corresponds well to the theoretical molecular weight of 16.6 kDa. The elution volume of ACHT1 from HiLoad 16/60 Superdex 200 indicated that ACHT1 exists as a monomer in solution (Fig. 1).

The initial crystals of ACHT1, which were found in condition No. 5 [2.0 M ammonium sulfate, 5%(v/v) 2-propanol] of Crystal Screen 2 within a week, were small and polycrystalline. After optimization, the best crystals, which were used for the

Figure 2
A crystal of ACHT1.**Table 3**
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL17U, SSRF
Wavelength (Å)	0.9793
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	1
Total rotation range (°)	120
Exposure time per image (s)	0.5
Space group	C222 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	102.7, 100.6, 92.8
α , β , γ (°)	90, 90, 90
Resolution range (Å)	50.00–1.70
Total No. of reflections	253890 (25632)
No. of unique reflections	52610 (5231)
Completeness (%)	99.4 (100)
Multiplicity	4.8 (4.9)
$\langle I/\sigma(I) \rangle$	23.2 (3.1)
$R_{\text{r.i.m.}}^{\dagger}$	0.072 (0.775)
CC _{1/2}	0.967 (0.854)

$\dagger R_{\text{r.i.m.}} = \sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th measurement of an equivalent reflection with indices *hkl* and where $N(hkl)$ is the total number of times that a given reflection is measured.

collection of diffraction data, were found in a condition consisting of 2.0 M ammonium sulfate, 8%(v/v) 2-propanol, 0.005%(w/v) agarose using the hanging-drop vapour-diffusion method with 13.5 mg ml⁻¹ protein at 277 K after a week (Table 2). The dimensions of the square crystals were about 0.1 × 0.1 × 0.02 mm (Fig. 2). Data were collected to 1.7 Å resolution (Fig. 3) at 100 K at SSRF. Preliminary diffraction analysis suggested that the crystals belonged to space group C222₁, with unit-cell parameters *a* = 102.7, *b* = 100.6, *c* = 92.8 Å (Table 3). Assuming the presence of two ACHT1 molecules per asymmetric unit, the Matthews coefficient was 2.97 Å³ Da⁻¹, which corresponds to 58.6% solvent content.

Similar structures have been reported, and it is expected that the structure of ACHT1 will be determined by molecular

Figure 3
A diffraction image from an ACHT1 crystal showing diffraction to a resolution of 1.7 Å.

replacement using these similar structures as search models. *Litopenaeus vannamei* Trx (PDB entry 3zzx; Campos-Acevedo *et al.*, 2013) may be useful as a search model as it shares 35% sequence identity with ACHT1. Elucidation of the crystal structure of ACHT1 will be of interest because it will be the first structure to be determined from the ACHT family, the functions of many members of which are unknown.

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