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Crystallization and preliminary X-ray analysis of 4-coumarate:CoA ligase from *Arabidopsis thaliana*

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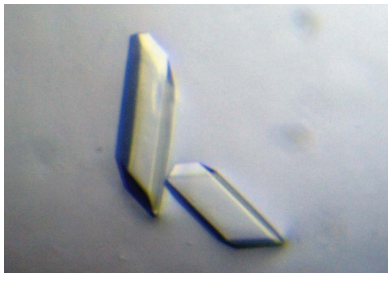

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4-Coumarate:CoA ligase 2 (4CL2) from *Arabidopsis thaliana* catalyzes the ATP-dependent formation of the 4-coumaroyl-CoA thioester through the formation of 4-coumarate-AMP. Recombinant 4CL2 protein was expressed in *Escherichia coli* and crystallized by the sitting-drop vapour-diffusion method. The crystals belonged to space group $P2_1$, with unit-cell parameters a = 91.6, b = 55.5, c = 124.4 Å, $\alpha = \gamma = 90.0$, $\beta = 111.1^\circ$.

1. Introduction

4-Coumarate:coenzyme A ligase (4CL; EC 6.2.1.12) plays a pivotal role in the biosynthesis of phenylpropanoids such as flavonoids and lignin in plants. The enzyme reaction proceeds by the initial adenylation of 4-coumaric acid by ATP and $MgCl_2$, which is followed by a thioester bond-forming reaction with CoASH and the concomitant release of 4-coumaroyl-CoA and AMP (Fig. 1; Knobloch & Hahlbrock, 1975). The activation of a carboxylic acid substrate by ATP to form an acyl-AMP intermediate is a common mechanism for adenylate-forming enzymes that catalyze different overall reactions, such as fatty acyl-CoA and aryl-CoA ligases, firefly luciferase and the adenylation domains of the modular nonribosomal peptide synthetases (NRPSs). These enzymes, which share at least ∼20% overall amino-acid sequence identity and conserved ATP-binding motif sequences, are structurally homologous and form the ANL (**A**cyl-CoA ligases, **N**RPS adenylation domains and **L**uciferase enzymes) superfamily of adenylating enzymes (Gulick, 2009).

4CL isoform 2 from *Arabidopsis thaliana* (At4CL2) is constitutively expressed in lignifying tissues and mainly contributes to lignin biosynthesis (Stuible & Kombrink, 2001; Schneider *et al.*, 2003, 2005). The substrate specificity for the carboxylic acid starter molecule has been well studied and it has been reported that At4CL2 selectively accepts 4-coumaric acid and caffeic acid as starter acids to produce the corresponding CoA thioesters. However, the enzyme does not activate sinapic acid, benzoic acid, phenylacetic acid or phenylalanine (Schneider *et al.*, 2005). Furthermore, site-directed mutagenesis studies of At4CL2 revealed the amino-acid residues that determine the starter acid specificity, which led to the generation of At4CL2 mutants that activate sinapic acid, a substrate that is normally not accepted by the wild-type At4CL2 enzyme (Schneider *et al.*, 2003). Recently, the X-ray crystal structures of *Populus tomentosa* 4CL1, which shares 68.2% amino-acid sequence identity with At4CL2, was reported in the apo form and in forms complexed with AMP and the intermediate analogue 5′-[3-(4-hydroxyphenyl)propyl]phosphate (APP; Hu *et al.*, 2010). To further clarify the structure and function of the 4CL enzymes, we expressed hexahistidine-fused recombinant At4CL2 protein in *Escherichia coli* and obtained good-quality crystals of the recombinant enzyme.

2. Experimental

2.1. Construction of the expression plasmid

Total RNA was obtained from three-week-old *A. thaliana* seedlings using an RNeasy Plant Mini Kit (Qiagen) and was reverse-

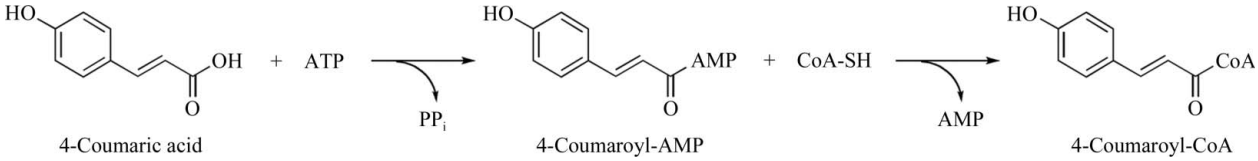

Figure 1
Proposed mechanism for the formation of 4-coumaroyl-CoA by At4CL2.

transcribed using SuperScript II Reverse Transferase (Invitrogen) with an oligo-dT primer (5′-GGCCACGCGTCGACTAGTACTT-TTTTTTTTTTTTTTT-3′). The first-strand cDNA mixture thus obtained was then used as the template to amplify the At1g02050 cDNA encoding full-length 4CL2 by PCR using 5′-ACGTACGC-ATGCATGACGACACAAGATGTGATAG-3′ as the sense primer, which introduces an *Sph*I restriction site, and 5′-GGTCAGCTGCA-GCTAGTTCATTAATCCATTTGCTAG-3′ as the antisense primer, which introduces a *Pst*I restriction site. The amplified DNA fragment was digested with *Sph*I/*Pst*I and ligated into the *Sph*I/*Pst*I sites of the pQE80L vector (Qiagen) for expression as a fusion protein with a His_6 tag at the N-terminus.

2.2. Expression and purification

After confirmation of the sequence, the pQE80L vector encoding full-length 4CL2 was transformed into *E. coli* M15. The cells harbouring the plasmid were cultured to an OD_{600} of 0.6 in LB medium containing 100 µg ml^{-1} ampicillin at 303 K. Isopropyl β-D-1-thiogalactopyranoside was then added to a final concentration of 1 m*M* to induce gene expression and the culture was incubated for a further 16 h at 303 K.

All of the following procedures were performed at 277 K. The *E. coli* cells were harvested by centrifugation at 5000*g* and resuspended in 50 m*M* Tris–HCl buffer pH 8.0 containing 0.2 *M* NaCl and 5%(*v*/*v*) glycerol (buffer *A*). The cells were disrupted by sonication and the lysate was centrifuged at 12 000*g* for 30 min. The supernatant was loaded onto an Ni Sepharose 6 Fast Flow column (GE Healthcare) equilibrated with buffer *A*. After washing the resin with 50 m*M* HEPES–NaOH buffer pH 7.0 containing 200 m*M* NaCl and 5%(*v*/*v*) glycerol (buffer *B*), the recombinant 4CL2 protein was subsequently eluted with buffer *B* containing 500 m*M* imidazole. The protein solution was then diluted fivefold with 50 m*M* HEPES–NaOH buffer pH 7.0 containing 5%(*v*/*v*) glycerol and 2 m*M* DTT (buffer *C*) and applied onto a Resource-Q column (GE Healthcare). The column was washed with buffer *C* containing 50 m*M* NaCl and the protein was subsequently eluted using a linear gradient of 50–300 m*M* NaCl. The protein solution was further purified to homogeneity by gel-filtration chromatography on Superdex 200 HR (10/300 GL; GE Healthcare) and concentrated to 10 mg ml^{-1} in 20 m*M* HEPES–NaOH pH 7.5 buffer containing 100 m*M* NaCl and 2 m*M* DTT.

A dynamic light-scattering (DLS) analysis was performed using a DynaPro-MSXTC molecular-sizing instrument (Protein Solutions Inc.). After centrifugation through an 0.22 µm Ultrafree-MC filter (Millipore) to remove particulate material from the protein solution, the solution properties of the purified protein were monitored. Data were acquired as 50 scattering measurements at 278 K and data from three sets of measurements were analyzed using the *DYNAMICS* software package (Protein Solutions Inc.) and averaged.

2.3. Crystallization and X-ray data collection

Initial crystallization attempts and optimization of the crystallization conditions were performed at 293 K using the sitting-drop vapour-diffusion method. 0.5 µl purified recombinant 4CL2 protein and an equal volume of reservoir solution were mixed and equilibrated against 100 µl reservoir solution using a 96-condition crystallization screen that was originally designed by Mitsubishi Chemical Corporation. Crystals were observed a few days later in a crystallization solution consisting of 100 m*M* MES–NaOH pH 6.5, 2 m*M* ATP and 24%(*w*/*v*) PEG 4000. Further crystallization was attempted by using Additive Screen (Hampton Research) at various pH values and by using 22%(*w*/*v*) PEG 4000 and 2 m*M* ATP as precipitants. Diffraction-quality crystals were finally obtained at 293 K in 100 m*M* MES–NaOH pH 6.0 containing 20% PEG 4000, 5 m*M* $MgCl_2$, 0.5 m*M* glutathione (GSH) and 2 m*M* ATP using the sitting-drop vapour-diffusion method (Fig. 2).

The crystals were transferred into a cryoprotectant consisting of 100 m*M* MES–NaOH pH 6.0, 20% PEG 4000, 5 m*M* $MgCl_2$, 0.5 m*M* GSH, 2 m*M* ATP and 20% glycerol. After a few seconds, the crystals were picked up in a nylon loop and then flash-cooled at 100 K in a nitrogen-gas stream. X-ray diffraction data sets were collected on beamline NW12 of Photon Factory-AR (wavelength 1.00000 Å) using an ADSC Quantum 210 CCD detector, with a distance of 130 mm between the crystal and the detector. A total of 180 frames were recorded with a 1° oscillation angle and 1 s exposure time. The data were indexed, integrated and scaled with the *HKL*-2000 program package (Otwinowski & Minor, 1997).

3. Results and discussion

The recombinant 4CL2 protein was expressed in *E. coli*. The purified His_6-tag-fused 4CL2 protein migrated as a single band with a molecular mass of 61 kDa on SDS–PAGE, which agrees well with the calculated value of 62.4 kDa. In contrast, gel-filtration analysis of the recombinant 4CL2 protein yielded a molecular mass of 65.9 kDa, suggesting that the recombinant 4CL2 protein exists as a monomeric enzyme. Furthermore, DLS analysis of the recombinant 4CL2 protein after gel filtration revealed a monomodal distribution, with a poly-

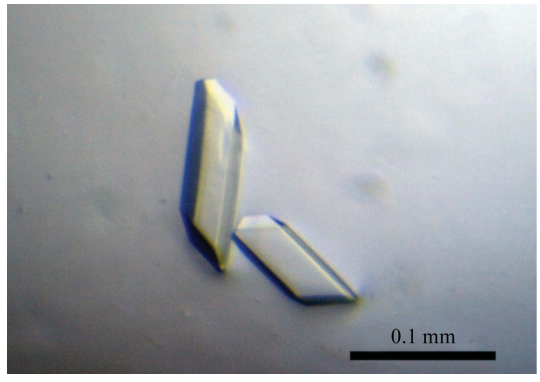

Figure 2
Crystal of 4CL2. The dimensions of the crystal were approximately 0.1 × 0.03 × 0.01 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Beamline	NW12, Photon Factory-AR
Wavelength (Å)	1.000
Temperature (K)	100
Detector	ADSC Quantum 210 CCD
Crystal-to-detector distance (mm)	130
Oscillation angle (°)	1
No. of frames	180
Exposure time (s)	1
No. of crystals	1
Space group	$P2_1$
Unit-cell parameters	
a (Å)	91.6
b (Å)	55.5
c (Å)	124.4
β (°)	111.1
Resolution (Å)	50.0–1.6 (1.63–1.60)
Total reflections	555709
Unique reflections	151778
Mosaicity (°)	0.45
Multiplicity	3.7 (3.6)
Completeness (%)	98.6 (98.5)
$\langle I/\sigma(I)\rangle$	40.4 (9.2)
$R_{\rm merge}$† (%)	3.4 (17.2)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl, $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

dispersity value of 26.6% and an estimated molecular mass of 52 kDa, also suggesting that the recombinant enzyme is monomeric.

Crystals appeared reproducibly within 3 d and the largest crystal grew to dimensions of approximately 0.1 × 0.03 × 0.01 mm (Fig. 2). A complete data set was collected to 1.6 Å resolution. Detailed data-processing statistics are shown in Table 1. Based on the diffraction data, the space group was determined to be $P2_1$, with unit-cell parameters a = 91.6, b = 55.5, c = 124.4 Å, $\alpha = \gamma$ = 90.0, β = 111.1°. With two monomers in the asymmetric unit, the Matthews volume ($V_{\rm M}$; Matthews, 1968) was calculated to be 2.4 Å^3 Da^{-1} and the estimated solvent content was 47.0%, which is in the range normally observed for protein crystals. A self-rotation function analysis using the *CNS* program (Brünger *et al.*, 1998) indicated that the twofold-symmetry axes are parallel to the crystallographic axes. Furthermore, a native Patterson function revealed the presence of a non-origin peak, which supports this conclusion. Structure determination by the molecular-replacement method is now under way using the *MOLREP* program (Vagin & Teplyakov, 2010) with the *A. thaliana* 4CL2 structure model generated by the *SWISS-MODEL* package (http://swissmodel.expasy.org/) based on the crystal structure of *P. tomentosa* 4CL (PDB code 3a9v; Hu *et al.*, 2010), which shares 68% identity with *A. thaliana* 4CL2, as a search model. Simultaneously, we are also attempting to crystallize the 4CL2 protein complexed with substrate and product analogues. These structural analyses will provide insights into not only the structure–-function relationship of 4CL2 but also the functional diversity of the ANL superfamily enzymes.

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