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Cloning, expression, purification and preliminary X-ray analysis of the dimerization domain of ethylene response sensor 1 (ERS1) from *Arabidopsis thaliana*

Ethylene signalling is initiated by a group of membrane-bound receptors with similarity to two-component systems. ERS1 belongs, together with ETR1, to subfamily 1, which plays a predominant role in ethylene signalling. The dimerization domain of ERS1 was crystallized in space groups $C222_1$ and $P2_12_12_1$, with two and four molecules per asymmetric unit, respectively. The crystals diffracted X-ray radiation to 1.9 Å resolution.

1. Introduction

Ethylene, a simple organic gaseous molecule, acts as a phytohormone. It controls a number of important developmental processes in plants such as germination, fruit ripening, senescence and responses to biotic and abiotic stresses (Bleecker & Kende, 2000). In *Arabidopsis thaliana*, the signal transduction pathway of ethylene is initiated by a group of five receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) which form disulfide-linked homodimers (Schaller *et al.*, 1995). Ethylene receptors are located in the endoplasmic reticulum membrane (Chen *et al.*, 2002), where the conserved hydrophobic N-terminal domain binds ethylene. The cytosolic part consists of a GAF domain, a histidine kinase (HK) domain which is composed of two subdomains, the dimerization (DHP) and catalytic (CA) domains, and a receiver domain (RD). The RD is absent in ERS1 and ERS2. The HK domain and RD show similarity to the bacterial two-component system (TCS; Chang *et al.*, 1993). TCS signalling comprises the signal-dependent autophosphorylation of a conserved His residue within the dimerization domain and the subsequent transfer of the phosphoryl group to a conserved Asp residue in the RD (Stock *et al.*, 2000). ETR1 and ERS1 exhibit HK activity (Moussatche & Klee, 2004) and possess all of the sequence motifs found in canonical HK domains. The remaining receptors lack one or more of these motifs and display Ser/Thr kinase activity *in vitro*, as does ERS1 under certain conditions (Moussatche & Klee, 2004). All of the receptors participate in ethylene signalling with overlapping roles (Hall & Bleecker, 2003), but ETR1 and ERS1 seem to play a predominant role. The receptors are active in the absence of ethylene and positively regulate the immediate downstream target CTR1, a putative Raf-like MAPK kinase kinase (MAPKKK; Kieber *et al.*, 1993), which inhibits the remaining pathway in its active form. Ethylene therefore acts as an inverse agonist of its pathway (Hua & Meyerowitz, 1998; Bleecker, 1999). There is evidence for an additional branch of the ethylene response pathway (Urao *et al.*, 2000) which follows the classical scheme of bacterial TCS signalling and which seems to play a role in the fine-tuning of the response (Hass *et al.*, 2004).

The exact role of the HK domain is still unclear, as the HK domain but not the kinase activity is needed for signalling (Qu & Schaller, 2004), while the latter is needed for growth recovery after ethylene withdrawal (Binder *et al.*, 2004). The effect of ethylene on ETR1 is controversial. It has been shown to inhibit (Voet-van-Vormizeele & Groth, 2008) and activate (Hall *et al.*, 2012) HK activity. Phosphorylation of the conserved histidine residue in the dimerization domain has been shown to regulate the interaction with EIN2, another crucial downstream participant of ethylene signalling (Bisson & Groth, 2010). In addition to the well known role in repressing the ethylene

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response, another role of ERS1 was reported to be in the promotion of the ethylene response in an ETR1-dependent manner (Liu *et al.*, 2010).

The ethylene signalling pathway presents an interesting case in which a two-component signalling system manipulates a MAPKKK and possibly a MAPKKK signalling cascade. While a number of structures of the HK domain and its subdomains are available from prokaryotes, no structural information is available from eukaryotes. Here, we report the purification, crystallization and preliminary X-ray crystallographic characterization of the ERS1 dimerization domain in two space groups.

2. Materials and methods

2.1. Cloning, expression and purification

The dimerization domain (DHP) of ERS1 (ERS1^{DHP}; residues 308–407) was amplified from a cDNA library obtained from the *Arabidopsis* Biological Research Centre at Ohio State University (Kieber *et al.*, 1993). The PCR protocol consisted of 25 cycles of annealing at 336 K followed by 10 s extension at 345 K using KOD polymerase (Novagen). The sequences of the forward and reverse primers were 5'-CAGGGCGCCAGTCATGCTGCAATTTTGGAAGAATCCATG-3' and 3'-GACCCGACGCGGTTAATCTTCCAATCTCGAAAGATCCAGAAC-5', respectively. Both primers contain appropriate extensions for ligation-independent cloning (LIC). The gene was inserted into a pETM-11/LIC vector (courtesy of A. Geerlof, Helmholtz Zentrum München) *via* LIC cloning. The vector contains an N-terminal His₆ tag followed by a TEV cleavage site. Owing to cloning, three additional amino acids (GAM) are added to the native sequence at the N-terminus after TEV proteolysis. The final inserts were verified by DNA sequencing.

The plasmid containing ERS1^{DHP} was transformed into *Escherichia coli* strain BL21 cells. Freshly transformed cells were used to inoculate 5 ml lysogeny broth (LB) medium (containing 50 µg ml⁻¹ kanamycin) and were grown overnight at 310 K. The overnight cultures were used to inoculate 2 l LB medium. They were grown to optical densities of between 0.6 and 0.7 at 600 nm, after which the temperature was lowered to 293 K. The bacteria were induced with 0.4 mM IPTG. Cultivation was continued for 18 h and the cells were harvested by centrifugation at 5500 rev min⁻¹ in a JLA-8.1000 rotor for 20 min at 277 K. The cell pellets were stored at 253 K. After thawing on ice, the pellets were resuspended in lysis buffer {20 mM

Tris pH 8.5, 250 mM NaCl, 10% glycerol, 20 mM imidazole, 1 mM Complete EDTA-free protease-inhibitor cocktail (Roche), 1 mg ml⁻¹ DNase (NEB), 0.1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Euromedex)} in a final volume of 20 ml per 5 g of cells and lysed by pulsed sonication on ice for 2 × 90 s. The lysates were centrifuged at 18 000 rev min⁻¹ in an SS-34 rotor at 277 K for 60 min. The supernatants were filtered through a 0.22 µm membrane and loaded onto a 5 ml Ni-NTA column which had first been equilibrated against 100 mM NiSO₄ in water and then against buffer A (20 mM Tris pH 7.5, 250 mM NaCl, 5% glycerol, 3 mM β-mercaptoethanol). The column was washed with 4 CV (column volumes) of buffer A followed by 3 CV of buffer A with 20% buffer B (buffer A with 250 mM imidazole). The proteins were eluted with a gradient of 20–100% buffer B in buffer A (50–250 mM imidazole) within 8 CV. Fractions containing ERS1^{DHP} were pooled and dialysed overnight at 277 K against buffer C (20 mM Tris pH 8.8, 150 mM NaCl, 6 mM β-mercaptoethanol) in a dialysis bag (molecular-weight cutoff 5 kDa) with 1 mg TEV protease (with a noncleavable N-terminal His tag) added per 30 mg of ERS1^{DHP}. The solution was collected after 16 h and passed again over the Ni-NTA column equilibrated against buffer C. The flowthrough was collected and concentrated using a Vivaspin column (molecular-weight cutoff 3 kDa). Size-exclusion chromatography (HiLoad 26/60 Superdex 75, Amersham Biosciences) was used as the final step of purification. The column was pre-equilibrated in buffer D (20 mM Tris pH 8.5, 150 mM NaCl). The samples eluted as a single peak corresponding to a molecular weight of 28 kDa, consistent with a dimeric state of the sample. The peak fractions were analysed by SDS-PAGE and pooled. The purity of the combined protein fractions was assessed with a 12% SDS-PAGE gel stained with Coomassie Brilliant Blue and the correct molecular weight was confirmed by mass spectrometry.

2.2. Crystallization

ERS1^{DHP} was concentrated to 12 mg ml⁻¹ using a Vivaspin column (molecular-weight cutoff 3 kDa). Initial crystallization trials were carried out with four different 96-well screens from Qiagen (The Classics, Classics II, PEGs and PEGs II Suites) at the EMBL Hamburg high-throughput crystallization facility (Mueller-Dieckmann, 2006). All initial screens were performed at 293 K in 96-well Greiner plates using the sitting-drop vapour-diffusion method. 300 nl protein solution was mixed with 300 nl reservoir solution and equilibrated against 50 µl reservoir solution.

Initial crystallization experiments with the His-tagged construct also yielded crystals, but they exhibited poor diffraction properties. Crystallization trials with tag-free ERS1^{DHP} resulted in five hits which mainly yielded needle clusters. Initial lead conditions of the tag-free variant were optimized through the use of the The Opti-Salts Suite (Qiagen), in which the original hit solution is mixed in a 9:1 ratio with different salts, buffers and other additives. Diffraction-quality crystals of ERS1^{DHP} were obtained at 293 K in space group *C*222₁ using 0.18 M L-proline, 0.1 M HEPES pH 7.5, 9% PEG 3350 (Fig. 1) and in space group *P*2₁2₁2 using 0.18 M L-proline, 0.1 M HEPES pH 7.5, 9% PEG 3350, 0.12 M sodium citrate.

2.3. X-ray diffraction data collection and processing

Prior to data collection, a single crystal of ERS1^{DHP} was briefly immersed in mother liquor augmented with 20% ethylene glycol as a cryoprotectant. The crystals were then flash-cooled to 77 K in liquid nitrogen. A complete X-ray diffraction data set was collected on beamline ID-29 at the ESRF (Grenoble, France) using a PILATUS 6M detector. A total of 1800 frames were collected with a rotation

Figure 1

Crystals of ERS1^{DHP} from *A. thaliana* in space group *C*222₁. The large crystal shown is about 150 × 40 × 40 µm in size.

range of 0.1° and a crystal-to-detector distance of 337.1 mm from a crystal belonging to space group $C222_1$ (Fig. 2). A total of 570 frames were collected with a rotation range of 0.2° and a crystal-to-detector distance of 286.9 mm from a crystal belonging to space group $P2_12_12$. The data were indexed and integrated using *XDS* (Kabsch, 2010) and scaled with *SCALA* (Winn *et al.*, 2011). Table 1 summarizes the data-collection and processing statistics.

3. Results and discussion

Expression of the ERS1 dimerization domain from *A. thaliana* resulted in soluble protein, which was purified in a three-step procedure applying affinity and size-exclusion chromatography (SEC) with very good final yields of 20 mg per litre of culture medium. The samples were at least 95% pure as estimated by SDS-PAGE.

Previous crystallization trials with an N-terminally truncated construct (residues 326–407) yielded reasonably sized hexagonal crystals (of up to 200 μm in diameter) which did not diffract beyond 5 Å resolution even after extensive optimization. The crystals also displayed some kind of packing disorder, reflected by a pseudo-dodecagonal symmetry in reciprocal space. Extension of the N-terminus by 18 residues yielded similar expression levels and resulted in five leads in the initial crystallization experiments. The removal of the His tag was beneficial and improved the habit of the crystals and the X-ray diffraction properties. In the sitting-drop setup, crystals grew within a week. In 24-well Linbro plates with hanging drops, crystal growth frequently ceased and the growth time increased to one month. Those crystals were of poor quality as judged by their diffraction power. Therefore, only sitting-drop plates were subsequently used for optimization. Crystals had a rod-like shape with dimensions of $150 \times 40 \times 40 \mu\text{m}$. The best diffracting crystals of ERS1^{DHP} were grown from 0.18 M L-proline, 0.1 M HEPES pH 7.5, 9% PEG 3350. The addition of 0.12 M sodium citrate caused a change to space group $P2_12_12$.

Figure 2
Diffraction image of an ERS1^{DHP} crystal in space group $C222_1$.

Table 1

X-ray data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$C222_1$	$P2_12_12$
X-ray source	ID-29, ESRF	ID-29, ESRF
Wavelength (Å)	0.923	0.923
Temperature (K)	100	100
Crystal-to-detector distance (mm)	337.13	286.91
Rotation range per image ($^\circ$)	0.1	0.2
Total rotation range ($^\circ$)	180	114
Unit-cell parameters (Å)	$a = 75.04, b = 98.74, c = 77.1$	$a = 65.91, b = 69.19, c = 108.25$
Resolution range (Å)	49.3–1.9 (2.00–1.90)	47.3–2.0 (2.11–2.00)
Observed reflections	151061 (24280)	134592 (19772)
Unique reflections	22944 (3593)	33054 (4746)
R_{merge} (%)	5.5 (79.9)	5.2 (77.3)
$R_{\text{p.i.m.}}$ (%)	2.5 (35.5)	3.0 (41.6)
Completeness (%)	99.4 (97.6)	98.2 (97.5)
$\langle I/\sigma(I) \rangle$	14.3 (2.5)	13.5 (2.2)
Multiplicity	6.6 (6.8)	4.1 (4.2)
Wilson B factor ($\text{\AA}^2$)	43.7	36.7
Optical resolution (Å)	1.61	1.67

Diffraction data for ERS1^{DHP} were indexed in space group $C222_1$ with unit-cell parameters $a = 75.04, b = 98.74, c = 77.1$ Å and in space group $P2_12_12$ with unit-cell parameters $a = 65.91, b = 69.19, c = 108.25$ Å. In space group $C222_1$ the highest likelihood is for two molecules per asymmetric unit, based on the Matthews coefficient (Matthews, 1968), with a solvent content of 61%, while for space group $P2_12_12$ four molecules per asymmetric unit are predicted, with a solvent content of 54%. There are no significant peaks in either native Patterson maps or in self-rotation functions, indicating the absence of translational symmetry or rotational symmetry non-parallel to the crystallographic axes.

This is the first report of the crystallization and preliminary X-ray analysis of an HK dimerization domain of eukaryotic origin. Crystals appeared in two different space groups after minor changes in the crystallization conditions, yielding data of comparable quality. This can be subsequently used to analyse possible influences of the crystal packing on the structure. Elucidation of the crystal structure of the dimerization domain of ERS1 by molecular replacement (MR) using the deposited structures of bacterial HK domains failed, despite a sequence identity of 36% to an HK domain from *Thermotoga maritima* (PDB entry 2c2a; Marina *et al.*, 2005). This might be caused by the nearly complete α -helical content of the domain, which is expected to fold into two long antiparallel helices. This may prevent the MR software placing the search model unambiguously. Selenomethionine-labelled protein is being expressed and purified and should allow subsequent experimental phasing.

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