Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Cloning, expression, purification and preliminary X-ray analysis of the protein kinase domain of constitutive triple response 1 (CTR1) from *Arabidopsis thaliana*

Hubert Mayerhofer,[a] Christoph Mueller-Dieckmann[b] and Jochen Mueller-Dieckmann[a]*

[a]EMBL Hamburg Outstation, c/o DESY, Notkestrasse 85, D-22603 Hamburg, Germany, and [b]ESRF, 6 Rue Jules Horowitz, F-38043 Grenoble CEDEX 09, France

Correspondence e-mail: jochenmd@embl-hamburg.de

Received 11 August 2010
Accepted 16 November 2010

Ethylene, a gaseous plant hormone, is perceived by a group of membrane-bound receptors. Constitutive triple response 1 (CTR1) from *Arabidopsis thaliana* directly interacts with ethylene receptors and thus links signal reception to the intracellular signalling pathway. The C-terminal protein kinase domain of CTR1 has been crystallized in its wild-type form and as a kinase-dead mutant. The wild-type crystals diffracted X-ray radiation to 3 Å resolution and the crystals of the kinase-dead mutant diffacted to 2.5 Å resolution. The crystals belonged to space groups $P4_12_12$ and $P4_22_12$, respectively, with two molecules per asymmetric unit in both cases.

1. Introduction

Plants synthesize ethylene, express it in most tissues and use it as a hormone (Wang *et al.*, 2002; Tsuchisaka & Theologis, 2004). Despite its simple character, this gaseous hydrocarbon regulates numerous effects in plants, such as growth, fruit ripening, senescence, seed germination and response to stress and pathogens (Bleeker & Kende, 2000). In *Arabidopsis thaliana*, five partly redundant membrane-bound receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) sense ethylene and initiate the signal-transduction pathway. Sequence analysis of ethylene receptors (ER) indicated a similarity to bacterial two-component systems (TCS) in their C-terminal cytosolic histidine kinase (HK) and receiver domains. However, only ETR1 and ERS1 contain all of the sequence motifs found in canonical HK domains and ERS1 and ERS2 lack the receiver domains. The receptors are active in the absence of ethylene *in vivo* and are inhibited upon ethylene binding (Hua & Meyerowitz, 1998).

The immediate downstream target of ethylene receptors in *A. thaliana* is constitutive triple response 1 (CTR1; Clark *et al.*, 1998), a putative Raf-like MAPK kinase kinase (Kieber *et al.*, 1993). Just like the ethylene receptors, CTR1 is a negative regulator of ethylene signalling. *In vivo* mutants of CTR1 with abolished protein kinase activity show a constitutive triple response (CTR), which is a hallmark of ethylene signalling (Huang *et al.*, 2003). Thus, ethylene acts as an inverse agonist which inhibits activity of its receptors, thereby relieving the inhibition of ethylene signalling by CTR1.

CTR1 interacts with ER *in vivo* through its N-terminal domain of unknown function. This association is independent of whether or not ethylene is bound to the receptors. The interaction recruits CTR1 to the endoplasmatic reticulum, which is the location of the ER (Gao *et al.*, 2003). While the N-terminal domain of CTR1 itself has no autoinhibitory effect *in vitro* on its C-terminal kinase domain (Huang *et al.*, 2003), the association of ER and CTR1 is required to activate CTR1 and thereby control the ethylene-signalling pathway (Gao *et al.*, 2003). Active CTR1 inhibits the MKK9–MPK3/MPK6 pathway preventing the activation of the key transcription factor EIN3 (Yoo *et al.*, 2008).

The precise mechanism of CTR1 activation by ER remains unclear. As a first step towards elucidating this important process in plants, we have expressed and crystallized the catalytic kinase domain of CTR1 in its active form and as a kinase-dead mutant.

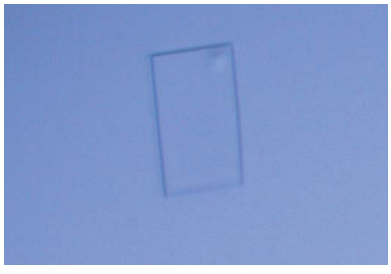

Table 1
X-ray data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	CTR1-kd	CTR1-D676N
X-ray source	ID23-2, ESRF	ID29, ESRF
Wavelength (Å)	0.8726	0.9763
Temperature (K)	100	100
Crystal-to-detector distance (mm)	293.3	371.2
Rotation range per image (°)	1.0	0.75
Total rotation range (°)	70	220
Space group	$P4_12_12$	$P4_22_12$
Unit-cell parameters (Å)	$a = b = 95.71$, $c = 179.78$	$a = b = 122.33$, $c = 95.01$
Resolution range (Å)	46.2–3.01 (3.18–3.01)	47.1–2.50 (2.64–2.50)
Observed reflections	93474 (14334)	334176 (52905)
Unique reflections	17053 (2648)	47817 (7636)
Multiplicity	5.5 (5.4)	13.1 (13.2)
R_{merge} (%)	17.6 (84.2)	9.0 (83.3)
$R_{p.i.m.}$ (%)	8.5 (40.0)	2.7 (25.3)
Completeness (%)	98.3 (97.4)	99.8 (98.9)
$\langle I/\sigma(I)\rangle$	9.5 (1.8)	16.9 (2.4)
Wilson B factor (Å^2)	73	60
Optical resolution (Å)	2.2	1.9

2. Materials and methods

2.1. Cloning, expression and purification

The C-terminal protein kinase domain of CTR1 (CTR1-kd, residues 540–821) was amplified from a cDNA library obtained from the Arabidopsis Biological Research Center at Ohio State University (Kieber *et al.*, 1993). The PCR protocol consisted of 25 cycles of annealing at 336 K followed by 20 s extension at 345 K using KOD polymerase (Novagen). The sequences of the forward and reverse primers were 5′-CAGGGCGCCATGGATGGTGATGATATGGACATCCCGTGGTGTGA-3′ and 5′-AGGCGGGTTGGCGAGCCTAAACATTGGCGCAGCCCAG-3′, respectively. Both primers contained appropriate extensions for ligation-independent cloning (LIC). The gene was inserted into a pETM-11/LIC vector (courtesy of A. Geerlof, EMBL Hamburg Outstation) *via* LIC cloning. The vector contains an N-terminal His_6 tag followed by a TEV cleavage site. Subsequently, the catalytically important and strictly conserved aspartate within the catalytic loop was mutated to asparagine using the QuikChange site-directed mutagenesis kit (Stratagene). This mutant, CTR1-D676N (residues 540–811), was produced after mass-spectrometric results indicated heterogeneous phosphorylation of CTR1-kd. This mutant also lacks ten amino acids at the C-terminus, which were removed in an attempt to improve crystallizability by various truncations at the termini. The sequences of the forward and reverse primers for CTR1-D676N were 5′-CAGGGCGCCAGTGATGGTGATGATATGGACATCCCGTG-3′ and 5′-CCTGAACGATTCTGGTAACTAGTTTAGTATTGGCGCAGCCCAG-3′, respectively. The final inserts were verified by DNA sequencing.

Plasmids containing CTR1-kd and CTR1-D676N were transformed into *Escherichia coli* strain BL21 cells co-expressing chaperones DnaK, DnaJ, GrpE, ClpB, GroEL and GroES. Freshly transformed cells were used to inoculate 5 ml Luria–Bertani (LB) medium (containing 50 µg ml^{-1} kanamycin) and were grown overnight at 310 K. The overnight cultures were used to inoculate 2 l of autoinduction medium (Studier, 2005). They were grown to optical densities of between 0.7 and 0.9 at 600 nm, after which the temperature was lowered to 293 K. 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 25 min at 277 K. The cell pellets were stored at 253 K. After thawing on ice, the pellets were resuspended in lysis buffer [20 m*M* HEPES pH 7.5, 250 m*M* NaCl, 20 m*M* imidazole, 3 m*M* β-mercaptoethanol (β-ME), 1 m*M* EDTA-free protease inhibitors, 1 mg ml^{-1} DNAse and 0.1%(*w*/*v*) CHAPS] 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} using 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 been equilibrated first against 100 m*M* $NiSO_4$ and then against buffer *A* (20 m*M* HEPES pH 7.5, 250 m*M* NaCl, 5% glycerol and 3 m*M* β-ME). The column was washed with four column volumes (CV) of buffer *A* followed by 3 CV of buffer *A* with 10% buffer *B* (buffer *A* containing 500 m*M* imidazole). The proteins were eluted with a gradient of 10–70% buffer *B* in buffer *A* (50–350 m*M* imidazole) within 8 CV and concentrated using a Vivaspin column (10 kDa molecular-weight cutoff). Size-exclusion chromatography (HiLoad 26/60 Superdex 75, Amersham Biosciences) was used as a final step of purification. The column was pre-equilibrated in buffer *D* [30 m*M* HEPES pH 7.5, 300 m*M* NaCl, 1%(*v*/*v*) glycerol and 1 m*M* DTT]. The samples eluted as a single peak with an apparent molecular weight of about 38 kDa, which is consistent with the calculated molecular weight of 34 kDa. Peak fractions were analyzed by SDS–PAGE and pooled. The purity of the combined protein fractions was assessed with a 4–20% gradient SDS–PAGE stained with Coomassie Brilliant Blue.

2.2. Crystallization

Wild-type CTR1-kd and CTR1-D676N were concentrated to 3 mg ml^{-1} using a Vivaspin column (10 kDa molecular-weight cutoff). The concentration was determined using a NanoDrop ND-1000 spectrophotometer from the absorption at 280 nm (extinction coefficient = 50 670 M^{-1} cm^{-1}). Prior to crystallization, AMPPNP with $MgSO_4$ (final concentration of 1 m*M*) or staurosporine (final concentration of 150 µ*M*), a generic protein kinase inhibitor, were added. Initial crystallization trials were carried out with four different 96-well screens from Qiagen (Classics I and II and PEGs I and II) at the EMBL Hamburg high-throughput crystallization facility (Mueller-Dieckmann, 2006). All initial screens were performed using the sitting-drop vapour-diffusion method at 293 K in 96-well Greiner plates. 400 nl protein solution was mixed with 400 nl reservoir solution and equilibrated against 50 µl reservoir solution.

Initial crystallization experiments resulted in several hits that produced small crystals (with approximate dimensions of 15 × 15 × 10 µm) of both CTR1-kd and CTR1-D676N. Crystals that were suitable for data collection were obtained using customized grid screens. The presence of staurosporine yielded crystals of superior size and habit when compared with those grown using AMPPNP. Diffraction-quality crystals of CTR1-kd were obtained at 293 K in 0.2 *M* K_2SO_4 and 15%(*w*/*v*) PEG 3350. The best crystals of CTR1-D676N grew from 1 *M* LiCl, 0.1 *M* citric acid pH 5.0 and 10%(*w*/*v*) PEG 6000.

2.3. X-ray diffraction data collection and processing

Prior to data collection, a single crystal of CTR1-kd or CTR1-D676N was briefly immersed in mother liquor augmented with 25% glycerol as a cryoprotectant. The crystals were then flash-cooled to 77 K in liquid nitrogen. Complete X-ray diffraction data sets were collected on beamlines ID23-2 and ID29 at the ESRF, Grenoble, France using a MAR 225 Mosaic CCD detector and an ADSC Q315R detector, respectively. For CTR1-kd a total of 170 frames were collected with a rotation range of 1.0°, while for CTR1-D676N a total of 220 frames were collected with a rotation range of 0.75°. The data were indexed and integrated using *XDS* (Kabsch, 2010) and were

scaled with *SCALA* (Collaborative Computational Project, Number 4, 1994). Table 1 summarizes the data-collection and processing statistics.

3. Results and discussion

Expression of CTR1-kd and CTR1-D676N from *A. thaliana* resulted in close to 100% soluble protein. Recombinant protein was purified in a two-step procedure, applying affinity and size-exclusion chromatography (SEC), to give final yields of 10 mg per litre of culture medium for each. Both samples eluted from the SEC column with an apparent molecular weight of 38 kDa, which was in agreement with the calculated molecular weight of the monomer of 34 kDa. The samples were at least 95% pure as estimated by SDS–PAGE.

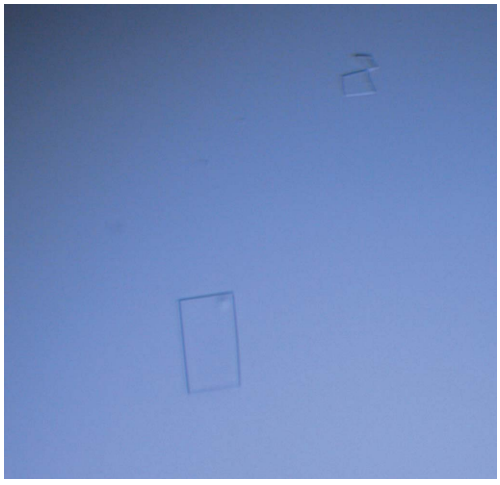

Figure 1
Crystals of CTR1-D676N from *A. thaliana*. The large crystal shown is about 70 × 40 × 15 µm in size.

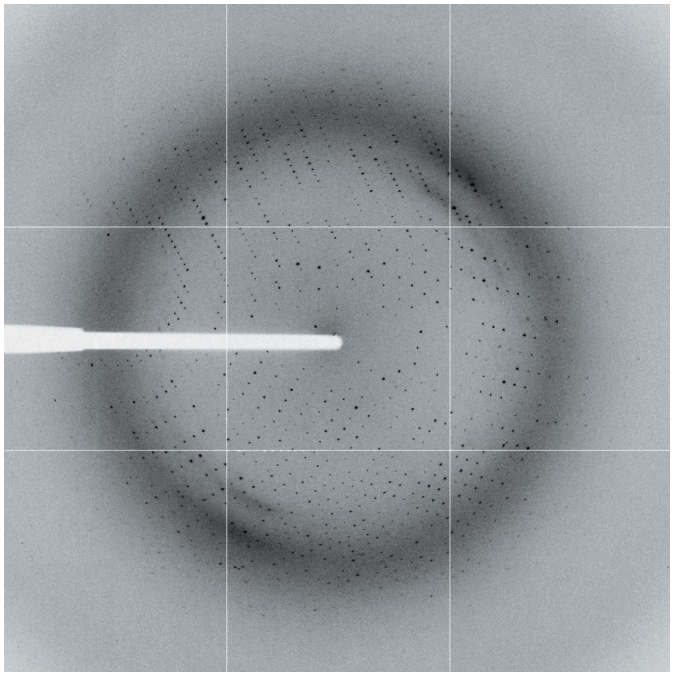

Figure 2
Diffraction image of a CTR1-D676N crystal.

The C-terminal domain of CTR1 shows similarity to the catalytic kinase domain of Raf1, which contains a number of phosphorylation sites. All of the phosphorylation sites of Raf1 within the catalytic kinase domain are activating (Dumaz & Marais, 2005). A mass-spectrometric analysis of wild-type CTR1-kd expressed in *E. coli* revealed the presence of between three and five phosphorylation sites. This finding is consistent with previous reports of autophosphorylation activity of CTR1 and CTR1-kd (Huang *et al.*, 2003). To our knowledge, however, the total number of phosphorylation sites and their precise locations within CTR1-kd remain unknown. At this point, we can only speculate which of the several species of multiply phosphorylated sample yielded crystals. Since crystals of CTR1-kd are sparse and fragile, attempts to wash a crystal for analysis *via* mass spectrometry were unsuccessful. In contrast to the heterogeneously phosphorylated wild-type sample, the kinase-dead mutant is not phosphorylated, as shown by MS.

Initial crystallization experiments resulted in several leads for CTR1-kd and CTR1-D676N. Even after extensive optimization trials, crystals of CTR1-kd grew infrequently and were difficult to reproduce. The diffraction quality of these crystals strongly correlated with the time of cryogenic preservation. Crystals appeared within about 24 h after setup and only those crystals which were frozen within 3–4 d of appearance diffracted X-ray radiation to about 3 Å resolution. Optimization of initial leads for CTR1-D676N converged quickly and the best crystals diffracted to 2.5 Å resolution. In both cases the initial crystallization conditions were improved using automated and manually produced customized screens. Crystals grown in the presence of staurosporine, a generic protein kinase inhibitor, were of superior size and habit when compared with those grown with AMPPNP. The best diffracting crystals of CTR1-kd were grown from 0.2 *M* K_2SO_4 and 15%(*w*/*v*) PEG 3350, while CTR1-D676N crystallized best from 1 *M* LiCl, 0.1 *M* citric acid pH 5.0 and 10%(*w*/*v*) PEG 6000 (Fig. 1).

The diffraction data of CTR1-kd were indexed in space group $P4_12_12$, with unit-cell parameters a = 95.7, c = 179.8 Å. During data reduction it became apparent that the crystal suffered from radiation damage, which was the reason for reducing the number of frames in the final data set to the first 70. The diffraction data of CTR1-D676N were indexed in the related space group $P4_22_12$, with unit-cell parameters a = 122.3, c = 95.0 Å (Fig. 2). In both cases the highest likelihood is two molecules per asymmetric unit, based on the Matthews coefficient (Matthews, 1968), with solvent contents of 58% for CTR1-kd and 53% for CTR1-D676N. There are no significant peaks in either native Patterson maps or in self-rotation functions, indicating an absence of translational symmetry and rotational symmetry nonparallel to the crystallographic axes.

Elucidation of the crystal structure of the C-terminal domain of CTR1 will be attempted by molecular replacement using a structure of the B-Raf family (38% sequence identity) as deposited with PDB code 3c4c (Tsai *et al.*, 2008) as a model.

We thank the Arabidopsis Biological Resource Center for providing us with an *A. thaliana* cDNA library. We acknowledge the ESRF for beam time on ID29 and ID23-2.

References

Bleeker, A. B. & Kende, H. (2000). *Annu. Rev. Cell Dev. Biol.* **16**, 1–18.
Clark, K. L., Larsen, P. B., Wang, X. & Chang, C. (1998). *Proc. Natl Acad. Sci. USA*, **95**, 5401–5406.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Dumaz, N. & Marais, R. (2005). *Mol. Cell*, **17**, 164–166.

Gao, Z., Chen, Y., Randlett, M. D., Zhao, X., Findell, J. L., Kieber, J. J. & Schaller, G. E. (2003). *J. Biol. Chem.* **278**, 34725–34732.
Hua, J. & Meyerowitz, E. M. (1998). *Cell*, **94**, 261–271.
Huang, Y., Li, H., Hutchison, C. E., Laskey, J. & Kieber, J. J. (2003). *Plant J.* **33**, 221–233.
Kabsch, W. (2010). *Acta Cryst.* D**66**, 125–132.
Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A. & Ecker, J. R. (1993). *Cell*, **72**, 427–441.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mueller-Dieckmann, J. (2006). *Acta Cryst.* D**62**, 1446–1452.
Studier, F. W. (2005). *Protein Expr. Purif.* **41**, 207–234.
Tsai, J. *et al.* (2008). *Proc. Natl Acad. Sci. USA*, **105**, 3041–3046.
Tsuchisaka, A. & Theologis, A. (2004). *Plant Physiol.* **136**, 2982–3000.
Wang, K. L.-C., Li, H. & Ecker, J. R. (2002). *Plant Cell*, **14**, S131–S151.
Yoo, S., Cho, Y., Tena, G., Xiong, Y. & Sheen, J. (2008). *Nature (London)*, **451**, 789–795.