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Cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of AHP2, a signal transmitter protein from *Arabidopsis thaliana*

Histidine-containing phosphotransfer proteins from *Arabidopsis thaliana* (AHP1–5) act as intermediates between sensor histidine kinases and response regulators in a signalling system called multi-step phosphorelay (MSP). AHP proteins mediate and potentially integrate various MSP-based signalling pathways (e.g. cytokinin or osmosensing). However, structural information about AHP proteins and their importance in MSP signalling is still lacking. To obtain a deeper insight into the structural basis of AHP-mediated signal transduction, the three-dimensional structure of AHP2 was determined. The AHP2 coding sequence was cloned into pRSET B expression vector, enabling production of AHP2 fused to an N-terminal His tag. AHP2 was expressed in soluble form in *Escherichia coli* strain BL21 (DE3) pLysS and then purified to homogeneity using metal chelate affinity chromatography and anion-exchange chromatography under reducing conditions. Successful crystallization in a buffer which was optimized for thermal stability yielded crystals that diffracted to 2.5 Å resolution.

1. Introduction

Two-component signalling pathways use phosphorylation on histidine and aspartate residues to sense and respond to a wide spectrum of environmental stimuli in bacteria (Stock *et al.*, 2000). The prototypical form of the two-component system (TCS) consists of a receptor with histidine kinase activity and a response regulator that triggers an appropriate downstream response, mostly regulating gene expression (Schaller *et al.*, 2008). In some bacteria, lower eukaryotes and plants, TCS has evolved into a modified system known as a multi-step phosphorelay (MSP). Compared with the prototypical TCS, the signal transduction in MSP includes two extra signalling modules (for recent reviews, see Horák *et al.*, 2011; To & Kieber, 2008). In comparison to the prototypical bacterial histidine kinase, the hybrid histidine kinase contains a response regulator-like receiver domain (RD) enabling the first phosphotransfer reaction to occur intramolecularly. An additional histidine phosphotransfer protein (Hpt) transmits the phosphate from the RD of membrane-localized histidine kinases to the nucleus, where it phosphorylates the final phosphate acceptor, response regulators (RR). In the genome of the model plant *Arabidopsis thaliana* there are eight histidine kinases (HKs), including AHK2, AHK3 and AHK4/CRE/WOL, which function as receptors for the plant hormone cytokinin (CK) (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006). ETR1 and ERS1 are members of the ethylene receptor family (Gamble *et al.*, 1998; Moussatche & Klee, 2004). However, the role of the histidine kinase activity of ETR1 and ERS1 is still not completely understood (Gamble *et al.*, 2002; Wang *et al.*, 2003), although it seems to play a rather minor role in ethylene signal transduction (Hall *et al.*, 2012). AHK1/AtHK1 appears to act as an osmosensor (Urao *et al.*, 2000; Tran *et al.*, 2007). CKI1 (Kakimoto, 1996; Pischke *et al.*, 2002; Hejatkó *et al.*, 2003, 2009; Deng *et al.*, 2010) and CKI2/AHK5 (Kakimoto, 1996; Iwama *et al.*, 2007; Desikan *et al.*, 2008) are cytokinin-independent histidine kinases. In addition to the sensor histidine kinases, the *Arabidopsis* genome encodes five Hpt proteins (AHP1–5; Miyata *et al.*, 1998; Suzuki *et al.*, 1998, 2000) containing a conserved histidine required for their ability to mediate phosphorelay. In addition, one pseudo-Hpt (APHP1/AHP6) was identified in

Arabidopsis that lacks the histidine phosphorylation site and acts as a negative regulator of CK signalling (Mähönen *et al.*, 2006).

Analyses of protein–protein interactions have shown that AHPs interact with several upstream (HKs) and downstream (ARRs, or *Arabidopsis* response regulators) signalling partners (Dortay *et al.*, 2006; Nakamura *et al.*, 1999; Urao *et al.*, 2000; Suzuki *et al.*, 2001; Pekárová *et al.*, 2011). It was suggested that AHPs probably function as a signalling hub and that redundancy appears to be an intrinsic attribute of MSP (Dortay *et al.*, 2006). This is also supported by several genetic analyses (Hutchison *et al.*, 2006; To *et al.*, 2004; Argyros *et al.*, 2008). However, recent studies have shown that there are also certain interaction preferences of the AHPs for histidine kinases. CKI1 hybrid histidine kinase prefers several members within the AHP family as interaction partners (Pekárová *et al.*, 2011). RD of CKI1 (CKI1_{RD}) is responsible for specific interactions with individual AHPs and CKI1_{RD} interacts with this specific subset of AHPs with different affinities (Pekárová *et al.*, 2011). Thus, these findings support the hypothesis that there is a certain level of specificity in signal transduction *via* the MSP system. However, the molecular mechanism by which the specificity among cognate partners in MSP is achieved is still not understood and is likely to depend on differences in amino-acid sequences and three-dimensional structures of both interaction partners. The structure of CKI1_{RD} has been determined (Pekárová *et al.*, 2011); however, the structure of the AHP proteins, which is necessary to uncover the structural determinants of CKI1-mediated MSP specificity, remains unknown.

Here, we report the cloning, protein production, purification, crystallization and preliminary X-ray diffraction analysis of AHP2, one of the HPT proteins from the model plant *A. thaliana*.

2. Materials and methods

2.1. Protein expression and purification

The AHP2 coding sequence was amplified from an *Arabidopsis* suspension culture cDNA library using an appropriate pair of primers containing an *Eco*RI restriction site. The sequences of the forward and reverse primers used were 5'-CCG **GAA TTC** ATG GAC GCT CTC ATT GCT CAG-3' and 5'-CCG **GAA TTC** TTA GTT AAT ATC CAC TTG AGG-3', respectively (the *Eco*RI restriction site is identified in bold). The amplified DNA was digested with *Eco*RI and ligated into the pRSET B vector (Invitrogen). The sequence of the resulting expression plasmid was verified by sequencing using an ABI 310 genetic analyser (Perkin Elmer, Norwalk, Connecticut, USA). It was then transformed into the *Escherichia coli* BL21 (DE3) pLysS expression strain (Novagen). The cells were grown in TB medium pH 7.5 supplemented with ampicillin (100 µg ml⁻¹) and 1% (w/v) glucose at 295 K. When an OD₆₀₀ of 1 was reached, gene expression was induced by adding isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.4 mM. 3 h after induction at 295 K, the cells were harvested by centrifugation at 3220g for 20 min at 277 K.

The cell pellets obtained from 1 l portions of culture were resuspended in 30 ml extraction buffer consisting of 50 mM Tris–HCl pH 7.9, 300 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol, 3.5 mM mercaptoethanol, 0.1% (v/v) Triton X-100 and sonicated applying 38 W pulses from a Sonicator 4000 (Qsonica, Newtown, Connecticut, USA). The cell lysate was then centrifuged at 47 448g for 30 min at 277 K to remove cell debris. The protein-containing supernatant was applied onto a HisTrap HP (5 ml) column (GE Healthcare) equilibrated in buffer consisting of 50 mM Tris–HCl pH 7.9, 300 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol, 3.5 mM β-mercaptoethanol. The protein was eluted using a gradient from buffer A [50 mM Tris–

HCl pH 7.9, 300 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol, 3.5 mM β-mercaptoethanol] to buffer B [50 mM Tris–HCl pH 7.9, 300 mM NaCl, 500 mM imidazole, 10% (v/v) glycerol, 3.5 mM β-mercaptoethanol].

The final purification was achieved by anion-exchange chromatography with a Resource Q column (GE Healthcare) equilibrated with 50 mM Tris–HCl pH 7.9, 5 mM DTT. The protein was eluted using a gradient of 0–2 M NaCl. The protein purity was determined by 15% SDS–PAGE followed by Coomassie Brilliant Blue staining and densitometry using a GS800 densitometer and *Quantity One 1-D Analysis* software (Bio-Rad Laboratories). Each protein fraction was concentrated to 5–10 mg ml⁻¹ using an Amicon Ultra-4 ultrafiltration cell with a 3 kDa cutoff (Millipore). The protein concentration was determined according to the Bradford protein assay (Bradford, 1976; Bio-Rad Laboratories) using bovine serum albumin as a calibration standard. AHP2 was stored at 193 K until further use.

2.2. Thermal shift assay

The optimal buffer composition for crystallization was determined using a thermal shift assay (Ericsson *et al.*, 2006). 22 buffers in the pH range 4.5–9 (Supplementary Table S1¹) were prepared for buffer-condition screening. Solutions of 0.1 mg ml⁻¹ purified and concentrated AHP2 protein, 100 mM appropriate buffer and 1000× diluted SYPRO Orange (Invitrogen) were mixed in the wells of a Fast 96-Well Reaction Plate (Applied Biosystems). To analyse the effect of reducing agent on AHP2 stability, 5 mM DTT was added to the reaction mixture. The plate was sealed with Optical Adhesive Cover (Applied Biosystems) and heated in a Step One Plus real-time PCR system (Applied Biosystems) from 293 to 363 K in increments of 0.2 K. Protein unfolding was monitored by the increase in the fluorescence of the SYPRO Orange probe using excitation and emission wavelengths of 490 and 575 nm, respectively. The thermal shift data were analysed using *Protein Thermal Shift* software v.1.0 (Applied Biosystems).

2.3. Crystallization

For crystallization, AHP2 (stored at 193 K) was thawed and transferred into 50 mM imidazole pH 8.0 with 5 mM DTT and concentrated again to 5 mg ml⁻¹ using 3 kDa Amicon Centrifugal Filter Units (Millipore). Initial screening experiments were performed in 24-well Clover Plates (Jena Bioscience) at 298 K using JBScreen Basics 1–4 (Jena Bioscience), The PACT Suite (Qiagen) and Crystal Screen and Crystal Screen 2 (Hampton Research) and the sitting-drop vapour-diffusion method. Generally, 2 µl protein solution was mixed with 2 µl reservoir solution and equilibrated against 800 µl reservoir solution. Small single crystals were obtained within 2 d in condition No. 20 of Crystal Screen 2 (0.1 M MES pH 6.5, 1.6 M MgSO₄).

2.4. Data collection and processing

Diffraction data have been collected on BL14.2 operated by the Joint Berlin MX-Laboratory at the BESSY II electron storage ring (Berlin-Adlershof, Germany) (Mueller *et al.*, 2012). The beamline is equipped with a Rayonics CCD 225 mm detector. Crystals were mounted in polymer microloops (Hampton Research, MiTeGen) directly from the crystallization drop and flash-cooled in a 100 K liquid-nitrogen stream without additional cryoprotection. Native diffraction data were recorded at a wavelength of 0.918 Å with an

¹Supplementary material is available from the IUCr electronic archives (Reference: GJ5115).

Figure 1
15% SDS-PAGE analysis of the purity of AHP2 used for crystallization. The protein was purified by metal chelate chromatography followed by anion-exchange chromatography. To determine the purification efficiency of AHP2, 30 μ l of each fraction (D1, D2, D3 and D4) eluted from the anion-exchange chromatography column was loaded into the well of the gel. The migration and size (in kDa) of the molecular-mass standards are indicated on the right. The expected molecular weight of AHP2 is 22.3 kDa.

Figure 2
Melting temperatures (T_m) in K for AHP2 in the presence of 100 mM buffers and in the absence or presence of reducing agent as measured by thermal shift assay.

oscillation angle of 0.5° per image, 5 s exposure time per image and a crystal-to-detector distance of 280 mm. The data set was indexed, integrated and scaled using the *XDSAPP* graphical user interface (Krug *et al.*, 2012) for running *XDS* (Kabsch, 2010). The space group was determined using *POINTLESS* (Evans, 2006). Matthews parameters were calculated with the program *MATTHEWS_COEF* (Matthews, 1968), which is part of the *CCP4* package (Winn *et al.*, 2011).

3. Results and discussion

The HPT AHP2 was cloned, overexpressed and purified to >95% purity according to densitometric analysis of Coomassie Brilliant Blue-stained SDS-PAGE gels (Fig. 1). Freshly isolated protein was concentrated to 5 mg ml⁻¹ and used for crystallization experiments. The initial crystallization conditions for AHP2 were identified using condition No. 20 of Crystal Screen 2 (Hampton Research) consisting of 0.1 M MES buffer pH 6.5, 1.6 M MgSO₄. Small tetragonal crystals which turned out to be very fragile were grown in 2 d at 298 K. However, these crystals did not diffract X-rays beyond 6.0 Å resolution. Further optimization of the discovered conditions included the screening of the pH, the incubation temperature and the type and concentration of the precipitant, but nothing led to a significant improvement of the crystal quality. In order to further expand the

Figure 3
Crystals of the AHP2 protein. The length of the scale bar is 100 μ m.

Figure 4
Diffraction image of the AHP2 crystals. The crystals diffracted to a resolution of 2.5 Å and showed significant anisotropic behaviour.

screening to protein buffer composition, a thermal shift assay was performed. This allowed the quick analysis of the thermodynamic stability of AHP2 as a function of pH and buffer composition. 13 of the assayed buffer conditions were not favourable for protein crystallization because they were represented by curves with high initial fluorescence background and no clear or very weak protein-unfolding transition (data not shown). The single sigmoidal shape of the melting curves obtained using the remaining nine buffer conditions allowed the evaluation of melting temperatures (T_m). These curves represented buffers in the pH interval 6.2–8 (Fig. 2), with melting temperatures ranging between 309 and 323 K. In most cases, the addition of 5 mM DTT slightly increased the T_m values (Fig. 2). A buffer composed of 50 mM imidazole pH 8.0 and 5 mM DTT was identified as the most stabilizing condition. In this condition AHP2 exhibited a melting temperature of 325 K (Fig. 2). The protein was

Table 1

Data-collection statistics for the crystals of AHP2.

Values in parentheses are for the highest-resolution shell.

No. of crystals	1
Beamline	HZB BL14.2
Detector	Rayonics MX-225
Wavelength (Å)	0.91841
Crystal-to-detector distance (mm)	280
No. of images	180
Oscillation per frame (°)	0.5
Resolution range (Å)	50.0–2.53 (2.68–2.53)
Space group	$P4_12_1$ or $P4_32_1$
Unit-cell parameters (Å, °)	$a = b = 59.3$, $c = 169.9$, $\alpha = \beta = \gamma = 90.0$
Measured reflections	75837
Unique reflections	10808
Mosaicity (°)	0.21
$R_{\text{meas}}^\dagger$ (%)	8.2 (101.4)
Completeness (%)	99.6 (98.7)
Multiplicity	7.02
$\langle I/\sigma(I) \rangle$	15.7 (1.8)

$^\dagger R_{\text{meas}}$ is the redundancy-independent merging R factor, also known as $R_{\text{r.i.m.}}$ (Diederichs & Karplus, 1997; Weiss & Hilgenfeld, 1997; Weiss *et al.*, 1998). $R_{\text{meas}} = \sum_{hkl} [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean of the $N(hkl)$ individual measurements $I_i(hkl)$ of the intensity of reflection hkl .

then transferred to this buffer prior to crystallization and simultaneously the crystallization temperature was lowered to 277 K. This optimization resulted in the formation of bipyramidal-shaped crystals with maximum dimensions of approximately $0.35 \times 0.2 \times 0.1$ mm grown within 1 week from a solution consisting of 100 mM MES pH 5.7–6.5, 1.6 M MgSO_4 (Fig. 3).

The results of the diffraction data-collection experiment of these crystals are summarized in Table 1. The crystal diffraction was enhanced from 6.0 to 2.5 Å resolution, albeit showing highly anisotropic behaviour (Fig. 4). As a consequence, the obtained value of R_{meas} was quite large, although the value of $\langle I/\sigma(I) \rangle$ remained reasonable (Table 1). The crystal belonged to the primitive tetragonal space group $P4_12_1$ (or $P4_32_1$), with unit-cell parameters $a = b = 59.34$, $c = 169.9$ Å. According to the Matthews coefficient ($V_M = 3.41 \text{ Å}^3 \text{ Da}^{-1}$), the crystals contained one molecule per asymmetric unit, which would correspond to a solvent content of 64% (Matthews, 1968).

Attempts to phase AHP2 by molecular replacement using HPT MtHPT1 from *Medicago truncatula* (PDB entry 3us6; M. Ruszkowski, K. Brzezinski, R. Jedrzejczak, M. Dauter, Z. Dauter, M. Sikorski & M. Jaskolski, unpublished work) as a search model have so far been unsuccessful. Consequently, we are currently attempting to solve the phase problem by heavy-atom derivatization.

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