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Crystallization and preliminary X-ray diffraction study of the histidine-containing phosphotransfer protein ZmHP1 from maize

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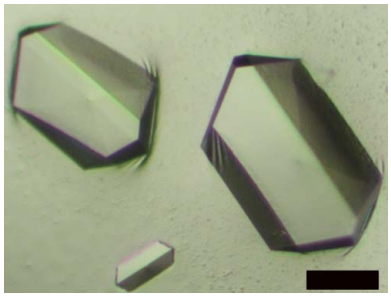
In histidine-aspartate phosphorelays (two-component systems) involved in plant-hormone signalling, histidine-containing phosphotransfer (HPt) proteins mediate the transfer of a phosphoryl group from the sensory histidine kinase to the response regulator. The maize HPt protein ZmHP1 has been crystallized. Although ZmHP1 with an N-terminal His tag could be crystallized using sodium chloride as a precipitant, the crystals diffracted poorly to only 3.2 Å resolution. When the His tag was removed, ZmHP1 crystals were obtained using polyethylene glycol 4000 as a precipitant and the diffraction data were greatly enhanced to 2.4 Å resolution. The crystals belonged to the space group $P4_12_12$, with one ZmHP1 molecule in the asymmetric unit.

1. Introduction

The His-Asp phosphorelay (a two-component system) is a signal transduction mechanism originally studied in prokaryotes (Stock *et al.*, 2000) and is also known to function in eukaryotes such as fungi and plants (Saito, 2001). In plants, the phosphorelay is triggered by the phytohormones ethylene and cytokinin, as well as by environmental stress (Hwang *et al.*, 2002). The signal cascade comprises a sensory histidine kinase (HK), a histidine-containing phosphotransfer (HPt) protein and a response regulator (RR). In response to extracellular or intracellular signals, HK autophosphorylates a His residue in the HK domain and the phosphoryl group is transferred to an Asp residue of the protein's own receiver domain. The phosphoryl group is transferred successively to a His residue of the HPt protein and an Asp residue of the receiver domain of RR. Phosphorylation of RR leads to signal output after interaction with target proteins or specific DNA motifs (Stock *et al.*, 2000; Saito, 2001). In *Arabidopsis thaliana*, 11, five and 22 genes for HKs, HPt proteins and RRs were identified, respectively (Kakimoto, 2003). In maize, three (*ZmHP1* to *ZmHP3*) and ten genes (*ZmRR1* to *ZmRR10*) for HPt proteins and RRs have also been identified (Sakakibara *et al.*, 1999; Asakura *et al.*, 2003). In *A. thaliana* and maize, physical interactions between specific HPt proteins and RRs have been demonstrated (Suzuki *et al.*, 2001; Asakura *et al.*, 2003), which suggests that each gene of plant HPt proteins should differentiate functionally. If so, the discrimination mechanisms at the molecular level are crucial for plant HPt proteins. We very recently determined the crystal structure of ZmHP2 (Sugawara *et al.*, 2005). However, detailed comparisons of all structures of the isoforms of ZmHPs will be essential to understand the discrimination mechanism. The sequence identity between ZmHP1 and ZmHP2 is only 65%, indicating that the homology model-based structure of ZmHP1 may not be reliable in attempting to clarify the mechanism of transfer. Thus, we crystallized the maize HPt protein ZmHP1 and performed a preliminary X-ray diffraction study. As a result of removing the His tag, tetragonal crystals were obtained that diffracted to 2.4 Å resolution.



2. Material and methods

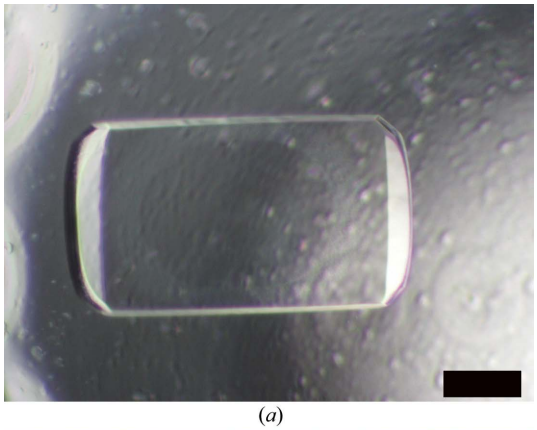
2.1. Protein expression and purification

Cloning, expression and purification of ZmHP1 proteins has been reported previously (Asakura *et al.*, 2003). The coding region of

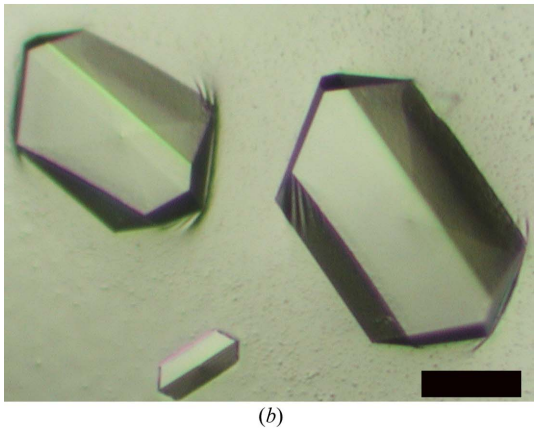
ZmHP1 was amplified by the polymerase chain reaction with the primers 5′-GGATCCATGTCTGCCGCGAACC-3′ and 5′-CTGCAGGTCCACTCCACCATC-3′. The *Bam*HI/*Pst*I fragment was ligated into pQE30 (Qiagen). The resulting plasmid was designated pQEZmHP1.

To remove the N-terminal His tag using dipeptidyl peptidase I, the original start Met site (ATG) was replaced by a Lys codon (AAG) using a QuikChange kit (Stratagene). The resulting plasmid was designated pQEZmHP1MK.

For the production of ZmHP1, *Escherichia coli* strain M15 (Qiagen) harbouring pREP4 (Qiagen) was used as the host cell. The pQEZmHP1 transformants were cultured with shaking in M9 medium supplemented with 1 *M* sorbitol, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1} kanamycin at 310 K until an A_{600} of 1.0 was obtained. The temperature of the medium was reduced to 298 K and isopropyl-D-thiogalactopyranoside was added to a final concentration of 1 m*M*. The culture was grown overnight with shaking. Cells were harvested and lysed with CelLytic B-II (Sigma). After treatment with DNase I, cell debris was removed by centrifugation at 18 000*g* for 20 min. The supernatant was loaded onto an Ni–NTA superflow column (Qiagen) equilibrated with 50 m*M* Tris–HCl pH 8.0, 0.3 *M* NaCl, 10%(*v*/*v*) glycerol and 3.5 m*M* 2-mercaptoethanol. Adsorbed proteins were eluted with 0.5 *M* imidazole. Gel filtration was performed on a HiLoad Superdex 75 prep-grade column (Amersham Biosciences) equilibrated in 25 m*M* Tris–HCl pH 7.5, 0.2 *M* NaCl and 3.5 m*M* 2-mercaptoethanol. The samples were dialyzed against 10 m*M* Tris–HCl pH 7.5 and 1 m*M* dithiothreitol.



(*a*)



(*b*)

Figure 1
Crystals of ZmHP1. The bars represent 0.2 mm. (*a*) His-tagged ZmHP1 crystals grown using sodium chloride as precipitant. (*b*) His-tag removed ZmHP1 crystals grown using PEG 4000 as precipitant.

Table 1
Diffraction data statistics.

Values in parentheses refer to the highest resolution shell.

	With His tag	Without His tag
Space group	$I4_122$	$P4_12_12$
Resolution limit (Å)	3.2 (3.31–3.2)	2.4 (2.53–2.4)
Total reflections	67966	56560
Unique reflections	10955	6763
$R_{\rm merge}$† (%)	8.2 (25.9)	6.0 (23.5)
Completeness (%)	97.7 (97.3)	95.0 (90.3)
Multiplicity	6.2 (4.8)	7.9 (8.1)
Mean $I/\sigma(I)$	8.7 (4.2)	8.9 (3.2)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I(hkl)_i - \langle I(hkl)\rangle| / \sum_{hkl} I(hkl)$, where $I(hkl)_i$ is the ith measurement of the intensity of reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity of reflection hkl.

The expression and purification conditions for non-His-tagged ZmHP1 were the same as for His-tagged ZmHP1, except that pQEZmHP1MK transformants were used instead of pQEZmHP1 transformants. The sample was digested using dipeptidyl peptidase I (Qiagen) to remove the His tag according to the suppliers' protocol. The enzymes and unprocessed ZmHP1 proteins were removed using an Ni–NTA superflow column. His-tag-removed samples were also dialyzed against 10 m*M* Tris–HCl pH 7.5 and 1 m*M* dithiothreitol.

2.2. Protein crystallization

Crystallization of both samples was carried out by the hanging-drop vapour-diffusion method. Initial screening for crystallization took place using the Crystal Screen 1 and 2 kits (Hampton Research). 2 µl of protein solution (10 mg ml^{-1}) was mixed with an equal volume of reservoir solution and was allowed to equilibrate against 0.5 ml reservoir solution.

2.3. Data collection and processing

All X-ray diffraction experiments were carried out using an R-AXIS IV imaging-plate area detector mounted on a Rigaku Ultrax-18 rotating-anode source with Cu $K\alpha$ radiation. All image data were processed with the programs *DPS* (Rossman & van Beek, 1999) and *SCALA* (Collaborative Computational Project, Number 4, 1994). Crystals of ZmHP1 with a His tag were mounted in quartz capillaries and subjected to X-ray diffraction. Crystals of ZmHP1 without a His tag were flash-frozen in a nitrogen cold stream.

3. Results and discussion

His-tagged ZmHP1 contains 156 residues with a molecular weight of 17.8 kDa. For ZmHP1, crystals were first observed using 2.0 *M* sodium chloride and 0.1 *M* sodium acetate buffer pH 4.6. After the crystallization conditions had been optimized, plate-like crystals (1.0 × 0.4 × 0.2 mm) were obtained after two weeks in 0.9 *M* sodium chloride and 0.1 *M* sodium acetate buffer pH 4.6 at 277 K (Fig. 1*a*). The space group was determined to be tetragonal $I4_122$, with unit-cell parameters $a = b = 91.33$, $c = 309.19$ Å. However, the crystals only diffracted to 3.2 Å resolution. The diffraction data statistics are summarized in Table 1.

To improve the diffraction quality of ZmHP1 crystals, the His tag was removed. ZmHP1 without a His tag is composed of 144 residues with a molecular weight of 16.4 kDa. ZmHP1 did not crystallize under the same crystallization conditions as ZmHP1 with a His tag. After re-screening the crystallization conditions, crystals were observed using 12%(*w*/*v*) polyethylene glycol (PEG) 20 000 and

0.1 *M* MES buffer pH 6.5. After the crystallization conditions had been optimized, square prismatic crystals (0.6 × 0.4 × 0.3 mm) were obtained after 1–2 d in 15–20%(*w*/*v*) PEG 4000, 0.1 *M* MES buffer pH 5.9–6.5 at 293 K (Fig. 1*b*). The crystals were also obtained with 20%(*w*/*v*) glucose as a cryoprotectant in the solution. The space group was determined to be tetragonal $P4_12_12$ (or its enantiomorph $P4_32_12$), with unit-cell parameters $a = b = 59.87$, $c = 100.32$ Å. Compared with the crystals of ZmHP1 with a His tag, the crystals of ZmHP1 without a His tag had greatly enhanced diffraction quality to 2.4 Å resolution. Assuming the presence of one molecule in the asymmetric unit, the value of the Matthews constant V_M (Matthews, 1968) is 2.75 Å^3 Da^{-1}, corresponding to a solvent constant of 54.9%; these are normal values for protein crystals. The diffraction data statistics are summarized in Table 1.

Preliminary molecular-replacement calculations for the non-His-tagged ZmHP1 crystal were performed with the program *AMoRe* (Navaza, 1994). The search model was based upon the ZmHP2 structure (Sugawara *et al.*, 2005). Both possible space groups were tested. Supposing that the space group was $P4_12_12$, a clear peak was found with a correlation coefficient (CC) of 0.373 and an *R* factor of 51.4% (10–4.5 Å) after translation-function calculations. The *R* factor was reduced to 50.2% and the CC increased to 0.426 (8–3 Å) after rigid-body refinement. Moreover, the placement of the model using the molecular-replacement solution shows plausible packing interactions. Thus, the space group was identified as $P4_12_12$. Structure refinement is currently in progress.

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