Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of *Saccharomyces cerevisiae* Ypd1p, a key intermediate in phosphorelay signal transduction

Myong Gyong Lee, Jae Young Lee, Hyun Kyu Song and Se Won Suh*

Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-742, South Korea

Correspondence e-mail: sewonsuh@plaza.snu.ac.kr

Received 1 January 1999
Accepted 11 March 1999

Ypd1p, a 167-residue protein from *Saccharomyces cerevisiae*, plays a key role in osmosensing phosphorelay signal transduction. It forms part of a multistep phosphorelay system which also includes Sln1p hybrid histidine kinase and two response regulators, Ssk1p and Skn7p. It has been overexpressed in soluble form in *Escherichia coli* with a His_6-tag at its C-terminus. The recombinant protein has been crystallized at room temperature using ammonium sulfate and lithium sulfate as precipitants. Native diffraction data have been collected to 2.3 Å using synchrotron radiation. The crystals are triclinic, belonging to the space group *P*1, with unit-cell parameters $a = 65.78$, $b = 66.74$, $c = 65.75$ Å, $\alpha = 106.60$, $\beta = 106.48$, $\gamma = 115.53^\circ$. The asymmetric unit contains four molecules of the monomeric recombinant Ypd1p, with a corresponding V_m of 2.75 Å^3 Da^{-1} and a solvent content of 55.3%.

1. Introduction

A signal-transduction strategy referred to as the 'two-component' regulatory system or His–Asp phosphorelay system is commonly employed by prokaryotic organisms in order to sense changes in their environment and to respond appropriately (Mizuno, 1998; Eggar *et al.*, 1997; Hoch & Silhavy, 1995). This signalling machinery, ubiquitous in bacteria, consists of two basic units: a sensor histidine kinase and a response regulator. More elaborate His–Asp–His–Asp four-step phosphorelay signal-transduction pathways have also been identified, such as the KinA/KinB/KinC–Spo0F–Spo0B–Spo0A system in *Bacillus subtilis* (Appleby *et al.*, 1996). However, it has been established that such phosphorelay systems also operate in eukaryotic organisms (Loomis *et al.*, 1997; Wurgler-Murphy & Saito, 1997; Swanson & Simon, 1994; Chang & Meyerowitz, 1994; Swanson *et al.*, 1994). The best characterized eukaryotic phosphorelay system is the His–Asp–His–Asp multistep phosphorelay, which governs osmoregulation in the budding yeast *Saccharomyces cerevisiae* (Posas *et al.*, 1996). This pathway responds to high osmolarity stress and lies upstream of a MAP kinase cascade, mediating changes in glycerol accumulation (Burg *et al.*, 1996). It involves the proteins Sln1p, Ypd1p and Ssk1p.

Sln1p, the sole transmembrane histidine kinase encoded by the genome of *S. cerevisiae*, is a hybrid or unorthodox histidine kinase which possesses both a conserved histidine-kinase (H1 module) and an aspartate-containing receiver (D1 module) response regulator domain (Ota & Varshavsky, 1993). Under normal growth conditions, it is active and autophosphorylates His576, from where the phosphate is relayed to Asp1114. The phosphate is then transferred to His64 of the phosphorelay intermediate Ypd1p (H2 module) and passed along to Asp554 of the Ssk1p response regulator (D2 module). The phosphotransferase Ypd1p thus binds to the receiver domains of both Sln1p and Ssk1p to mediate the multistep phosphorelay. Phosphorylation of the C-terminal receiver domain in the Ssk1p response regulator renders it unable to activate the MAP kinase cascade, which is composed of three tiers of protein kinases, namely Ssk2p/Ssk22p MAPKKKs, Pbs2p MAPKK and Hog1p MAPK (Eggar *et al.*, 1997; Whitmarsh & Davis, 1998). Specific phosphatases which act at the level of the MAP kinase cascade have also been identified (Eggar *et al.*, 1997). At high osmolarity, the histidine kinase Sln1p is no longer active and the unphosphorylated Ssk1p leads to an enhanced level of intracellular glycerol, so that cells can continue to grow in the hyperosmotic environment. The Sln1p osmosensor may not specifically sense an external osmolyte but is tied to osmotic balance by an unknown mechanism (Tao *et al.*, 1999). In *S. cerevisiae*, there is also a second high-osmolarity sensor Sho1p which is non-essential and is not a histidine kinase. Instead, it has an SH3 domain which interacts with the N-terminal proline-rich domain of Pbs2p MAPKK (Maeda *et al.*, 1995; Ruis & Schüller, 1995).

Recently, another signal-transduction pathway has been identified for the osmosen-

sing histidine kinase Sln1p in *S. cerevisiae*. A second response regulator, Skn7p, is also influenced by Sln1p and its partner Ypd1p (Ketela *et al.*, 1998). The data strongly imply that phosphotransfer from Sln1p to Ypd1p to Skn7p (Asp427) is occurring. Similar results by Li *et al.* (1998) establish that the Sln1p-regulated osmotic stress pathway is bifurcated, with Sln1p kinase transmitting signals *via* Ypd1p to two response regulators, Ssk1p and Skn7p. Therefore, Ypd1p plays a key role in this increasingly complex signal-transduction pathway in *S. cerevisiae*.

The phosphorelay intermediate Ypd1p is an interesting target for structural studies, since it is known to functionally interact with at least three different receiver domains. To date, three-dimensional structures of only two bacterial counterparts have been determined: the C-terminal HPt domain of *Escherichia coli* ArcB (Kato *et al.*, 1997) and Spo0B response regulator phosphotransferase from *B. subtilis* (Varughese *et al.*, 1998). Both structures are composed of a basic α-helix bundle unit, but the former is a monomer whereas the four-helix bundle in the latter is formed by dimerization of the two subunits through a parallel association of helices. These results suggest that H2 module phosphotransferases of markedly different tertiary and quaternary structures may serve a common function in phosphorelay signal transduction. Crystallization of a complex between the C-terminal HPt domain of *E. coli* ArcB and the chemotaxis response regulator CheY has also been reported (Kato *et al.*, 1998). In comparison, structural data on eukaryotic phosphorelay H2 module phosphotransferases are not available. In order to provide such information, we have initiated the structure determination of Ypd1p, a 167-residue protein in *S. cerevisiae*, which plays a key role in the osmosensing phosphorelay signal transduction. As the first step, well diffracting crystals have been produced. Here, we report preliminary X-ray crystallographic data as well as the crystallization conditions.

2. Experimental

2.1. Protein expression and purification

The gene encoding Ypd1p was amplified by PCR using the *S. cerevisiae* genomic DNA as a template. The amplified DNA was inserted into the *Nde*I/*Xho*I-digested expression vector pET-22b. This vector construction, designated as pET-22b-Ypd1p, adds six histidine residues to the C-terminus of the gene product to facilitate protein purification. The complete nucleotide sequence of the insert was confirmed by dideoxy-DNA sequencing performed at the Research Center for Microbiology, Seoul National University. The enzyme was highly overexpressed in soluble form in B834(DE3) cells upon induction by 0.2 m*M* IPTG at 310 K. Cells were grown in LB medium for 4 h after IPTG induction and were harvested by centrifugation at 7000 rev min^{-1} (Sorvall GSA rotor) for 7 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer [20 m Tris–HCl pH 7.9, 0.50 *M* NaCl, 50 m*M* imidazole and 1 m*M* PMSF] and was then homogenized by sonication. The crude lysate was centrifuged at 36 000*g* (18 000 rev min^{-1}, Hanil Supra 21K rotor) for 30 min at 277 K and the recombinant protein in the supernatant fraction was purified by three chromatographic steps. The first step, utilizing the C-terminal histidine tag, was metal-chelate chromatography on Ni–NTA resin (Qiagen). Next, gel filtration was performed on a HiLoad XK 16 Superdex 75 prep-grade column (Pharmacia), which had previously been equilibrated with buffer *A* (20 m*M* Tris–HCl pH 7.5) containing 150 m*M* sodium chloride. Further purification was achieved by an ion-exchange chromatographic step on Source 15Q resin packed in a HR 10/10 column (Pharmacia), which had previously been equilibrated with buffer *A*. Prior to loading the protein sample on this ion-exchange column, the salt concentration was lowered to 50 m*M* by diluting it with buffer *A*. The protein was eluted with a linear gradient of 0–1.0 *M* sodium chloride in buffer *A*. The purified Ypd1p was homogeneous, as judged by polyacrylamide gel electrophoresis in the presence of 0.1%(*w*/*v*) sodium dodecyl sulfate (Laemmli, 1970). This procedure yielded approximately 55 mg of homogeneous Ypd1p from a 2 l culture. The purified protein was dialyzed against buffer *A* containing 100 m*M* sodium chloride, and the protein solution was concentrated using a YM2 membrane (Amicon). The protein concentration was estimated by measuring the absorbance at 280 nm, employing a correspondence of 1 mg ml^{-1} concentration to an A_{280} of 0.8 for the 1 cm path length. For the overexpression of selenomethionine-substituted protein, M9 cell-culture medium containing extra amino acids was used instead of LB medium and 30 m*M* β-mercaptoethanol was added immediately after metal-chelate chromatography on Ni–NTA resin. The purified protein was dialyzed against buffer *A* containing 100 m*M* sodium chloride and 10 m*M* dithiothreitol (DTT) and the protein solution was concentrated using a YM2 membrane (Amicon).

2.2. Dynamic light-scattering studies

The dynamic light-scattering experiment was performed on a Model DynaPro-801 instrument from Protein Solutions (Charlottesville, Virginia). The data were collected at room temperature from 2 mg ml^{-1} protein in buffer *A* containing 100 m*M* sodium chloride.

2.3. Crystallization

Crystallization was achieved by the hanging-drop vapour-diffusion method at 296 K using 24-well tissue-culture plates (Flow Laboratories). A hanging drop was prepared by mixing equal volumes (4 µl each) of the protein solution and the reservoir solution. The protein concentration was 23 mg ml^{-1} prior to mixing with the reservoir solution. Each hanging drop was placed over 0.5 ml reservoir solution. Initial crystallization conditions were established by sparse-matrix sampling (Jancarik & Kim, 1991).

2.4. X-ray diffraction experiment

A crystal was mounted in a thin-walled glass capillary and the capillary was sealed with wax after filling both ends with the mother liquor. Initial X-ray experiments were carried out using graphite-monochromated Cu $K\alpha$ X-rays from a rotating-anode generator (Rigaku RU-200BH) running at 40 kV and 70 mA with a 0.3 mm focus cup. The first set of X-ray diffraction data was collected at 293 K on a FAST area-detector system (Enraf–Nonius) using the *MADNES* software (Messerschmidt & Pflugrath, 1987). The unit-cell parameters were determined by the autoindexing and parameter-refinement procedures of *MADNES*. The reflection intensities were obtained by the profile-fitting procedure (Kabsch, 1988) and the data were scaled by the Fourier scaling program (Weissman, 1982). The second set of native X-ray data was collected at 100 K using a Weissenberg camera for macromolecular crystallography at the BL-6A experimental station of the Photon Factory, Japan (Sakabe, 1991). The synchrotron X-ray wavelength was 1.000 Å and a 0.2 mm collimator was used. A Fuji image plate (20 × 40 cm) was placed at a distance of 429.7 mm from the crystal. The oscillation range per image plate was 5.5°, with a speed of 2.0° s^{-1} and a coupling constant of 1.0° mm^{-1}. An overlap of 0.5° was allowed between two contiguous image

Table 1
Synchrotron data-collection statistics.

Number of crystals	1
Temperature (K)	100
Space group	*P*1
Unit-cell parameters (Å, °)	a = 65.78, b = 66.74, c = 65.75 , α = 106.60, β = 106.48, γ = 115.53
Number of measured reflections	52869
Number of unique reflections	36851
$R_{\rm merge}$† (%)	6.7
Data completeness (%)	79.3 (30–2.3 Å), 61.8 (2.4–2.3 Å)

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

plates. Diffraction patterns recorded on the image plates were digitized with a Fuji BA100 scanner. The raw data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results

The native molecular mass of 24 kDa, as estimated by the dynamic light-scattering analysis, indicated that the recombinant Ypd1p from *S. cerevisiae* with the C-terminal His_6-tag (calculated M_r = 20 099) exists as a monomer with a polydispersity of 21%. Well diffracting crystals of native protein were obtained when the reservoir solution [100 m*M* HEPES–KOH, 1.4 *M* ammonium sulfate, 0.7 *M* lithium sulfate and 2%(*v*/*v*) PEG 400 at final pH 7.36] was prepared by mixing appropriate volumes of 1.0 *M* HEPES–KOH (pH 7.35), 4.0 *M* ammonium sulfate, 2.0 *M* lithium sulfate and 100% PEG 400, and adjusting the final volume with water. The crystals grew to maximum dimensions 0.8 × 0.5 × 0.3 mm within two weeks (Fig. 1).

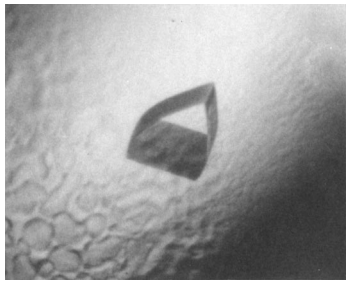

Figure 1
A triclinic crystal of *S. cerevisiae* Ypd1p. Its approximate dimensions are 0.5 × 0.4 × 0.2 mm.

The native crystals diffracted to 2.3 Å resolution with Cu $K\alpha$ X-rays from a rotating-anode source and were very stable in the X-ray beam. The first set of diffraction data were collected at 293 K from a native crystal using Cu $K\alpha$ radiation. A total of 15 796 unique reflections were measured with a redundancy of 1.9. The merged data set is 89.2% complete to 2.9 Å and gives an $R_{\rm merge}$ (on intensity) of 6.4%. The crystals belong to the triclinic space group *P*1, with unit-cell parameters a = 66.98, b = 66.89, c = 67.02 Å, α = 106.02, β = 107.00, γ = 115.65°. The selenomethionine-substituted protein crystallized isomorphously under similar crystallization conditions to the native protein but in the presence of 20 m*M* DTT, and had unit-cell parameters a = 66.80, b = 66.82, c = 66.79 Å, α = 105.86, β = 106.83, γ = 115.91°. Data were collected to 3.6 Å, but the Se atoms could not be located from the isomorphous difference Patterson map. The second set of native X-ray data was collected at 100 K using synchrotron X-rays. A total of 52 869 measured reflections were merged into 36 851 unique reflections with an $R_{\rm merge}$ (on intensity) of 6.7%. The merged data set is 79.3% complete to 2.3 Å, with the shell completeness between 2.4 and 2.3 Å being 61.8%. Table 1 summarizes the statistics for the synchrotron data collection. The self-rotation function calculation indicated the presence of four molecules of recombinant Ypd1p in the crystallographic asymmetric unit, giving a V_m of 2.75 Å^3 Da^{-1} and a solvent content of 55.3%. These values are within the frequently observed ranges for protein crystals (Matthews, 1968). The electron-density map obtained by the multiple isomorphous replacement method reveals a mostly α-helical structure, which will be described elsewhere.

Note added in proof: After submission of this manuscript, an independent crystallization of Ypd1p in a tetragonal space group has been reported (Xu *et al.*, 1999).

We thank Professor N. Sakabe, Dr N. Watanabe, Dr M. Suzuki and Dr N. Igarashi for assistance during data collection at BL-6A of Photon Factory, Japan. We also thank the Inter-University Center for Natural Science Research Facilities, Seoul National University for providing the X-ray equipment. This work was supported by grants from the Korea Science and Engineering Foundation through the Center for Molecular Catalysis at Seoul National University and Korea Ministry of Education (BSRI-98-3418). HKS is supported by the Postdoctoral Fellowship from Korea Ministry of Education.

References

Appleby, J. L, Parkinson, J. S. & Bourret, R. B. (1996). *Cell*, **86**, 845–848.
Burg, M. B., Kwon, E. D. & Últz, D. (1996). *FASEB J.* **10**, 1598–1606.
Chang, C. & Meyerowitz, E. M. (1994). *Res. Microbiol.* **145**, 481–486.
Eggar, L. A., Park, H. & Inouye, M. (1997). *Genes Cells*, **2**, 167–184.
Hoch, J. A. & Silhavy, T. J. (1995). Editors. *Two-Component Signal Transduction.* Washington, DC: ASM Press.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kabsch, W. (1988). *J. Appl. Cryst.* **21**, 916–924.
Kato, M., Mizuno, T. & Hakoshima, T. (1998). *Acta Cryst.* D**54**, 140–142.
Kato, M., Mizuno, T., Shimizu, T. & Hakoshima, T. (1997). *Cell*, **88**, 717–723.
Ketela, T., Brown, J. L., Stewart, R. C. & Bussey, H. (1998). *Mol. Gen. Genet.* **259**, 373–378.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Li, S., Ault, A., Malone, C. L., Raitt, D., Dean, S., Johnston, L. H., Deschenes, R. J. & Fassler, J. S. (1998). *EMBO J.* **17**, 6952–6962.
Loomis, W. F., Shaulsky, G. & Wang, N. (1997). *J. Cell Sci.* **110**, 1141–1145.
Maeda, T., Takekawa, M. & Saito, H. (1995). *Science*, **269**, 554–558.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Messerschmidt, A. & Pflugrath, J. W. (1987). *J. Appl. Cryst.* **20**, 306–315.
Mizuno, T. (1998). *J. Biochem.* **123**, 555–563.
Ota, I. M. & Varshavsky, A. (1993). *Science*, **262**, 566–569.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–325.
Posas, F., Wurgler-Murphy, S. M., Maeda, T., Witten, E. A., Thai, T. C. & Saito, H. (1996). *Cell*, **86**, 865–875.
Ruis, H. & Schüller, C. (1995). *Bioessays*, **17**, 959–965.
Sakabe, N. (1991). *Nucl. Instrum. Methods A*, **303**, 448–463.
Swanson, R. V., Alex, L. A. & Simon, M. I. (1994). *Trends Biochem. Sci.* **19**, 485–490.
Swanson, R. V. & Simon, M. I. (1994). *Curr. Biol.* **4**, 234–237.
Tao, W., Deschenes, R. J. & Fassler, J. S. (1999). *J. Biol. Chem.* **274**, 360–367.
Varughese, K., Madhusudan, Zhou, X. Z., Whiteley, J. M. & Hoch, J. A. (1998). *Mol. Cell*, **2**, 485–493.
Weissman, L. (1982). *Computational Crystallography*, edited by D. Sayre, pp. 56–63. Oxford University Press.
Whitmarsh, A. J. & Davis, R. J. (1998). *Trends Biochem. Sci.* **23**, 481–485.
Wurgler-Murphy, S. M. & Saito, H. (1997). *Trends Biochem. Sci.* **22**, 172–176.
Xu, Q., Nguyen, V. & West, A. H. (1999). *Acta Cryst.* D**55**, 291–293.