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Purification, crystallization and preliminary X-ray diffraction analysis of the yeast phosphorelay protein YPD1

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YPD1 is a yeast osmoregulatory protein that functions in a phosphorelay signal-transduction pathway. YPD1 has been expressed in *Escherichia coli*, purified to homogeneity and crystallized. The crystals were obtained by hanging-drop vapor-diffusion using PEG 4000 as a precipitant. Preliminary X-ray diffraction analysis indicates that the crystals belong to tetragonal space group $P4_32_12$ or $P4_12_12$ with unit-cell dimensions $a = b = 52.71$, $c = 244.02$ Å. X-ray data to 2.7 and 3.0 Å have been collected from native crystals and a heavy-atom derivative, respectively. Positions for two Hg atoms have been located by analysis of difference Patterson maps.

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1. Introduction

YPD1 participates in a multi-component signaling pathway in *Saccharomyces cerevisiae* that regulates cellular responses to osmostress (Posas *et al.*, 1996). This regulatory pathway utilizes a phosphorelay mechanism involving three proteins, SLN1, YPD1 and SSK1, that have sequence and functional similarities to the large number of bacterial proteins known as two-component signal transducers (Maeda *et al.*, 1994; Ota & Varshavsky, 1993; Posas *et al.*, 1996). SLN1 is a transmembrane sensor kinase that catalyzes autophosphorylation of a specific histidine residue in response to external osmotic pressure. This phosphoryl group can then be transferred to an aspartic acid residue within the C-terminal regulatory domain of SLN1 (designated SLN1-R1). In a YPD1-dependent step, the phosphoryl group can be transferred from SLN1-R1 to an aspartic acid residue within the C-terminal domain of SSK1 (designated SSK-R2). This is achieved *via* formation of a phosphorylated-YPD1 protein intermediate. SSK1, in its phosphorylated state, negatively regulates the activity of a mitogen-activated protein (MAP) kinase cascade. Under conditions of hyper-osmotic stress, SSK1 becomes dephosphorylated, resulting in activation of the HOG1-dependent MAP kinase cascade and increased production of glycerol in the cell which restores the osmotic balance (Albertyn *et al.*, 1994; Brewster *et al.*, 1993; Maeda *et al.*, 1994, 1995).

YPD1 was found to have a distant relationship by sequence alignment (less than 20% amino-acid sequence identity) with proteins or functional domains in other two-component signaling pathways, such as the P1 domain of the bacterial chemotaxis histidine kinase CheA (Zhou *et al.*, 1995), the HPt domain of the anaerobic sensor kinase ArcB of *Escherichia coli* (Kato *et al.*, 1997) and the Spo0B protein involved in *Bacillus subtilis* sporulation (Burbulys *et al.*, 1991). Structural comparisons of the bacterial histidine-containing phosphotransfer domains of CheA and ArcB (Zhou *et al.*, 1995; Kato *et al.*, 1997) revealed a common four-helix bundle motif. It will be interesting to see whether this motif has been conserved in the yeast phosphorelay protein YPD1. During transfer of the phosphoryl group from SLN1 to SSK1, YPD1 becomes transiently phosphorylated on a specific histidine residue, His64 (Posas *et al.*, 1996; Janiak-Spens *et al.*, 1999). Thus, YPD1 can be considered as an essential phosphoprotein intermediate during phosphoryl transfer from SLN1 to SSK1. YPD1 must therefore interact with, and discriminate between, the two homologous regulatory domains associated with SLN1 (R1) and SSK1 (R2).

Although structural information is available for several prokaryotic two-component proteins, it is not known to what extent the emerging class of eukaryotic two-component regulatory proteins resemble their bacterial counterparts. Interestingly, a bacterial response-regulator protein CheY can serve as a phosphoryl donor to YPD1 *in vitro* (Janiak-Spens *et al.*, 1999), thus raising the question concerning conservation of form and function between the prokaryotic and eukaryotic kingdoms. Structural studies of YPD1 have been initiated in order to obtain a detailed picture of the environment surrounding the site of phosphorylation (His64), to identify molecular surfaces that are used for recognition and interaction with response-regulator domains, and to combine this knowledge with information from biochemical studies in order to gain insights regarding the phosphorelay mechanism.

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Table 1
Summary of X-ray data collection.

Values in parentheses are for the highest resolution shell.

	Native I (RT)	Native II (103 K)	$Hg(CH_3COO)_2$ (RT)
Resolution range (Å)	30.0–2.7	30.0–2.7	30.0–3.0
Unique reflections	8893	9451	6660
Mean I/σ	9.7 (11.2)	6.9 (6.9)	9.1 (6.3)
R_{sym}†	0.045 (0.057)	0.072 (0.086)	0.063 (0.094)
Completeness (%)	87.9 (77.6)	96.3 (90.5)	90.6 (90.1)
Multiplicity	2.8 (2.2)	4.1 (2.9)	3.4 (3.3)
R_{deriv}‡	—	—	0.29

† $R_{sym} = \sum(|I - \langle I \rangle|)/\sum I$, where I is the observed intensity and $\langle I \rangle$ is the mean intensity of symmetry-equivalent reflections. ‡ $R_{deriv} = \sum|F_{PH} - F_P|/\sum|F_P|$, where F_{PH} is the structure-factor amplitude for the derivative crystal and F_P is that of the room-temperature native crystal.

2. Materials and methods

2.1. Expression and purification

In order to obtain sufficiently large quantities of protein for structural studies, YPD1 was overexpressed and purified from *Escherichia coli* using a modified pUC12 vector, pME43 (Simms *et al.*, 1987). A DNA fragment containing the 501 base-pair gene encoding YPD1 was amplified by the polymerase chain reaction (PCR) using the following synthetic oligonucleotide primers obtained from Gibco-BRL: the 5′ primer 5′-TCGCTTACCAT**ATG**TCTACTATTCC-GTCAG-3′ and the 3′ primer 5′-CTAG-CTGCAGATTATAGGTTTGTGTTGTA-ATATTTAG-3′. For convenience in subcloning, the 5′-primer contained an *Nde*I site (underlined) which overlaps with the codon for the initiator methionine (bold) and the 3′-primer contained a *Pst*I site (underlined). Yeast genomic DNA, kindly provided by Dr Dan Riggs (University of Oklahoma), was used as a template in the PCR reaction using *Pfu* DNA polymerase (Stratagene). The resulting PCR product was digested with *Nde*I and *Pst*I. The parent vector, pME43, was also digested with *Nde*I and *Pst*I to remove a 1.3 kb internal fragment. The 2.7 kb pUC-vector was gel purified and then ligated to the YPD1 gene fragment overnight at 288 K using T4 DNA ligase (New England Biolabs). DH5α cells were transformed and the desired recombinant plasmid, designated pVN1, was obtained.

A 1 l culture of pVN1-containing DH5α cells was grown overnight at 310 K in Luria broth supplemented with 50 µg ml^{-1} ampicillin. The following purification steps were all carried out at 277 K. Cells were harvested by centrifugation at 4500g for 8 min and the pellet resuspended in 0.1 M sodium phosphate pH 7.0, 1 mM EDTA and 1.4 mM β-mercaptoethanol (βME). Cells were lysed by sonication and unbroken cells removed by centrifugation at 8000g. The supernatant was further clarified by ultracentrifugation at 100 000g.

Saturated ammonium sulfate was added slowly to the supernatant to a final concentration of 55% saturated ammonium sulfate. The protein precipitate was collected by centrifugation at 12 000g and then resuspended in approximately 5 ml of 50 mM Bis–Tris pH 6.5, 1.4 mM βME. The sample was dialyzed for 24 h against 2 l of the same buffer (with one buffer change) in order to remove ammonium sulfate.

The dialyzed sample was filtered and then applied to a 5 ml HiTrap Q Sepharose Fast Flow (Pharmacia) anion-exchange column pre-equilibrated in starting buffer (50 mM Bis–Tris pH 6.5). Protein was eluted from the column using a linear salt gradient of 0–500 mM NaCl in starting buffer. Fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). YPD1-containing fractions were combined and then concentrated to approximately 8 ml using an Amicon ultrafiltration device with a YM10 membrane. The sample was loaded onto a Sephadex G50 (Sigma) gel-filtration column (350 ml bed volume) equilibrated in 50 mM sodium phosphate pH 7.0, 1 mM EDTA, 1.4 mM βME. Fractions containing pure YPD1 protein were identified by SDS–PAGE and pooled. Protein concentration was determined by absorbance at 280 nm using a calculated extinction coefficient for YPD1 of 15280 M^{-1} cm^{-1}.

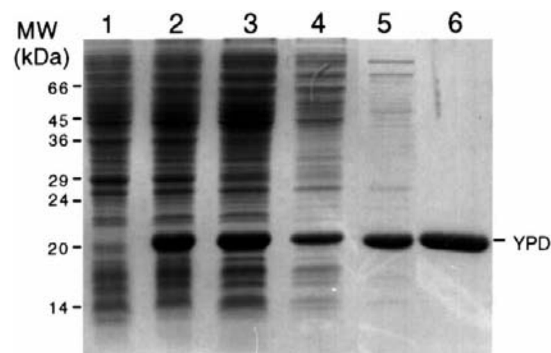

Figure 1
Purification of YPD1. Samples were removed at various stages during purification and analyzed by 15% SDS–PAGE. Lane 1, pUC12-containing DH5α whole-cell lysate (negative control); lane 2, pVN1-containing DH5α whole-cell lysate; lane 3, soluble cell extract after sonication; lane 4, sample following ammonium sulfate fractionation; lane 5, sample after Q Sepharose anion-exchange column; lane 6, sample from pooled fractions after Sephadex G50 gel-filtration column. Molecular-weight markers (kDa) are indicated to the left.

2.2. Crystallization

For crystallization trials using the sparse-matrix screening approach (Jancarik & Kim, 1991), the protein was dialyzed against 50 mM sodium acetate pH 6.5, 1 mM EDTA, 1.4 mM βME, concentrated to 12–15 mg ml^{-1} using Centricon 10 filter units (Amicon) and then filter-sterilized to remove any particulate matter. The first crystals were obtained at room temperature by hanging-drop vapor-diffusion using Hampton Research Crystal Screen I reagents (only #10 resulted in crystals). Further optimization of the crystallization conditions yielded reproducible diffraction-quality crystals using polyethylene glycol 4000 (Fluka) as a precipitant. In the vapor-diffusion setup, the reservoir was composed of 30–33%(w/v) PEG 4000, 0.1 M sodium acetate pH 5.0 and 0.2 M ammonium acetate. The hanging drop consisted of 1.5 µl of 10–12 mg ml^{-1} protein solution and 1.5 µl of reservoir solution.

2.3. Data collection and analysis

Data were collected at both room temperature and 103 K using an R-AXIS IIc image-plate system mounted on a Rigaku rotating-anode X-ray generator (Cu $K\alpha$ radiation) operated at 50 kV and 100 mA. The crystal-to-detector distance was 170 mm, and 0.5° oscillation images were taken with an exposure time of 10 min each. For data collected at 103 K, the crystal mother liquor served as a sufficient cryoprotectant such that crystals could be flash frozen directly in the N_2 stream or frozen prior to data collection in liquid propane and stored in liquid N_2 (Rodgers, 1997). The data were indexed and integrated using *DENZO* (Otwinowski, 1993) and then

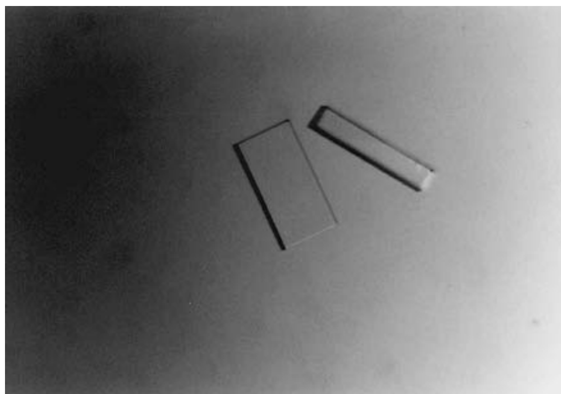

Figure 2
Tetragonal crystals of YPD1. Crystals were grown at room temperature by hanging-drop vapor diffusion using a reservoir containing 32%(*w*/*v*) PEG 4000, 0.1 *M* sodium acetate pH 5.0 and 0.2 *M* ammonium acetate. The hanging drop (3.0 µl) consisted of equal volumes of purified YPD1 protein (12 mg ml^{-1}) and reservoir solution.

scaled and merged with *SCALA* (Evans, 1993). Subsequent calculations were performed with the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994).

For preparation of the heavy-atom derivative, a concentrated stock solution of mercuric acetate (Sigma) was prepared in artificial mother liquor. A YPD1 crystal was then soaked for approximately 3 h at room temperature in the presence of approximately 4 m*M* mercuric acetate. The crystal was then mounted in a quartz capillary for data collection.

3. Results and discussion

High-level constitutive expression of the 19.2 kDa yeast YPD1 protein was achieved in *Escherichia coli* (Fig. 1, lane 2). Purification of YPD1 required three steps: ammonium sulfate fractionation, anion-exchange and gel-filtration column chromatography (Fig. 1, lanes 4, 5 and 6, respectively). The final product was judged to be >98% homogeneous by SDS–PAGE. The yield from 1 l of bacterial culture was approximately 50 mg of pure YPD1.

The protein was crystallized at room temperature from polyethylene glycol 4000. The initial crystals were large in size but exhibited internal and surface defects. Several factors affecting crystal growth were investigated to improve the quality of the crystals: pH, protein concentration, PEG of different molecular weight and concentration, additives and temperature. The optimized conditions (cited in §2) yielded diffraction-quality crystals of average dimensions 0.15 × 0.25 × 0.4 mm, as shown in Fig. 2, with good overall size and morphology.

The crystals belong to a tetragonal space group ($P4_32_12$ or $P4_12_12$) with unit-cell dimensions $a = b = 52.71$, $c = 244.02$ Å at room temperature and $a = b = 52.16$ and $c = 241.50$ Å when frozen at 103 K. The space group was assigned based on examination of extinction patterns for reflections $l = 4n$ and $h = 2n$. A calculated Matthews' volume (Matthews, 1968) of 2.1 Å^3 Da^{-1} is consistent with two molecules in the asymmetric unit with a corresponding solvent content of approximately 43%. There is no evidence for dimerization of YPD1, thus the two molecules in the asymmetric unit may represent independent monomers.

Table 1 summarizes the X-ray diffraction data that have been obtained thus far. Data were collected on R-AXIS IIc image-plate systems as guest users at the University of Texas Southwestern Medical Center and at the Rutgers University/UMDNJ X-ray facility. Native data sets were collected at both room temperature (RT) and at 103 K to the limits of the detector, which corresponds to a resolution of 2.7 Å. Although the crystals diffract very well (to 2.0 Å), due to the long cell dimension along *c*, we have not been able to collect high-resolution data beyond 2.7 Å on standard laboratory instruments.

A self-rotation function search did not give a clear orientation of a non-crystallographic twofold axis in the $\kappa = 180°$ plane. However, in a native Patterson map, a significant peak was observed near (0.5, 0.5, 0.0) which suggests that the two monomers in the asymmetric unit may be related by translational symmetry.

Data to 3.0 Å has been collected from a mercurial heavy-atom derivative (Table 1). From analysis of difference Patterson vectors, we have identified the location of two mercury-binding sites (presumably corresponding to the single cysteine residue in each monomer). We are currently screening for additional isomorphous heavy-atom derivatives to obtain more phase information.

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