Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Co-crystallization of the yeast phosphorelay protein YPD1 with the SLN1 response-regulator domain and preliminary X-ray diffraction analysis

Lilian Chooback and Ann H. West*

Department of Chemistry and Biochemistry, University of Oklahoma, 620 Parrington Oval, Norman, OK 73019, USA

Correspondence e-mail: awest@chemdept.chem.ou.edu

Received 26 December 2002
Accepted 25 February 2003

The SLN1, YPD1 and SSK1 proteins function in a multi-step phosphorelay signal transduction pathway in yeast. YPD1, a histidine-containing phosphotransfer (HPt) protein, mediates the transfer of a phosphoryl group between the two response-regulator domains associated with SLN1 and SSK1, the R1 and R2 domains, respectively. Co-crystallization of the SLN1-R1 domain with YPD1 is reported here. Two different crystal forms were obtained by the hanging-drop vapor-diffusion method using 2.6 *M* ammonium sulfate as a precipitant. X-ray diffraction analysis indicates that crystal form I belongs to a trigonal space group $P3_2/P3_1$, with unit-cell parameters $a = b = 91.4$, $c = 201.1$ Å, while crystal form II belongs to an orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 51.6$, $b = 74.2$, $c = 98.7$ Å. Complete data sets to 2.1 and 2.3 Å resolution have been collected from trigonal and orthorhombic crystals, respectively. Protein gel analysis indicates that in both crystal forms YPD1 and the SLN1-R1 domain are present in a 1:1 stoichiometry suggestive of a complex.

1. Introduction

In *Saccharomyces cerevisiae*, an expanded 'two-component'-based signal transduction system helps to control cellular responses to hyperosmotic stress (Maeda *et al.*, 1994; Ota & Varshavsky, 1993; Posas *et al.*, 1996; Saito, 2001). The plasma-membrane-bound SLN1 protein is composed of an N-terminal extracellular sensory domain, a central cytoplasmic histidine kinase (HK) domain and a C-terminal response-regulator domain (SLN1-R1). The SLN1 autokinase activity is modulated by external osmotic conditions. A phosphoryl group can be transferred from a conserved histidine residue within the HK domain to a conserved aspartyl side chain within the SLN1-R1 domain. The phosphorelay protein YPD1 is then required for phosphoryl group transfer from the SLN1-R1 domain to the phosphoreceiver domain on SSK1 (SSK1-R2). The phosphorylation state of the SSK1 response regulator determines the activity of the downstream HOG1-dependent MAP kinase cascade, which in turn affects the transcription of genes involved in the osmotic stress response.

The X-ray structure of YPD1 has been reported previously (Song *et al.*, 1999; Xu & West, 1999) and is composed of six α-helices and a short 3_{10}-helix. A four-helix bundle (helices *B*, *C*, *D* and *G*) forms the central core of the molecule, with the site of phosphorylation, His64, located in the middle of the αC helix. Using alanine-scanning mutagenesis coupled to a yeast two-hybrid interaction screen, we identified a hydrophobic patch on the surface of YPD1 as the binding site for the SSK1 response-regulator domain (Porter *et al.*, 2003). A similar interaction screen was carried out using YPD1 and the SLN1-R1 domain and the results suggest that the same hydrophobic patch is involved in general recognition of response-regulator proteins in yeast (S. P. Porter & A. H. West, unpublished results). We have initiated X-ray crystallographic studies in order to obtain direct information at the level of atomic resolution regarding protein–protein interactions involving the YPD1 phosphorelay protein. Here, we report co-crystallization of YPD1 with its upstream phosphodonor, the SLN1-R1 domain, in two different crystal forms.

2. Materials and methods

2.1. Protein purification

YPD1 was purified using the method developed by Xu *et al.* (1999). SLN1-R1 was overexpressed in *Escherichia coli* as a tripartite fusion protein using the IMPACT system (New England Biolabs) as described by Janiak-Spens *et al.* (1999). The SLN1-R1–intein–CBD fusion protein was purified from sonicated cell lysates using a 3 ml chitin bead affinity column equilibrated in lysis buffer (20 m*M* Tris–HCl pH 7.5, 500 m*M* NaCl, 1 m*M* EDTA). After sample loading, the column was washed sequentially with 20 column volumes of lysis

buffer and ten column volumes of cleavage buffer (20 m*M* Tris–HCl pH 7.5, 50 m*M* NaCl, 5 m*M* $MgCl_2$). Finally, the column was washed with five column volumes of cleavage buffer containing 30 m*M* β-mercaptoethanol. Buffer flow was stopped and the column was incubated overnight at 277 K to allow thiol-induced cleavage of the 15.3 kDa SLN1-R1 domain from the rest of the fusion protein. SLN1-R1 was then eluted from the column using cleavage buffer and further purified using a 300 ml bed volume Sephadex G50 (Sigma) gel-filtration column equilibrated in 20 m*M* Tris–HCl pH 7.5, 50 m*M* NaCl, 1 m*M* EDTA, 1 m*M* β-mercaptoethanol. Aliquots of the eluted protein sample were electrophoresed on a sodium dodecylsulfate polyacrylamide gel (SDS–PAGE) and fractions containing pure SLN1-R1 were pooled. The collected fractions were concentrated using an Amicon bioseparation system and a PAN/PVC ultrafiltration membrane with a molecular-weight cutoff of 10 kDa. 1–2 mg of SLN1-R1 protein per liter of cell culture was obtained and the protein was judged to be $\geq$99% pure by SDS–PAGE. The purified protein was stored at 193 K in gel-filtration buffer plus 10% glycerol.

2.2. Crystallization

The purified SLN1-R1 and YPD1 proteins were each concentrated to 10–12 mg ml^{-1}. With the aim of forming a beryllium fluoride–protein complex, the SLN1-R1 protein aliquot was incubated for 5 min at room temperature with 4 m*M* $MnCl_2$ and 45 m*M* NaF. $BeCl_2$ was added to a final concentration of 8 m*M* and the mixture was incubated for 15 min. YPD1 was then added and incubation was continued for another 15 min. The final protein concentration had SLN1-R1 present at a slight molar excess relative to YPD1 (approximately 1.3:1). This mixture was used immediately to set up crystal trays or could be stored for up to one week at 277 K.

Concentrated stocks of ammonium sulfate solutions were prepared at pH values of 4, 5, 6 and 7. The hanging-drop vapor-diffusion crystallization screen involved varying the ammonium sulfate concentration in the reservoir from 2–3 *M* in 0.2 *M* increments in the presence of 50 m*M* sodium acetate pH 5.3. The hanging drops consisted of 1.6 µl of the YPD1/SLN1-R1 protein mixture (with or without beryllium fluoride) and 1.3 µl of reservoir solution.

2.3. X-ray data collection and analysis

X-ray data were collected at 100 K using an R-AX1S IV^{++} image-plate detector mounted on a Rigaku RU-H3R X-ray generator operated at 50 kV and 100 mA. Crystals were transferred to artificial mother liquor containing 15% glycerol and then flash-frozen in an N_2-gas stream just prior to data collection. For form I crystals, the crystal-to-image-plate distance was 220 mm (for low-resolution data collection) or 180 mm (for high-resolution data collection) and 0.5° oscillation images were taken with an exposure time of 8 min. For form II crystals, the crystal-to-image-plate distance was set to 100 mm and 1° oscillation images were collected with an exposure time of 10 min. The data were indexed and integrated using *DENZO* (Otwinowski, 1993) and then scaled and merged with either *SCALEPACK* (Otwinowski & Minor, 1997) or *d*TREK* (Pflugrath, 1999).

2.4. Analysis of crystal content

10–12 medium-size crystals of each crystal form were pooled and rinsed with the equilibrated reservoir solution from the crystallization tray. The crystals were solubilized in SDS–PAGE loading buffer and the protein sample was then subjected to electrophoresis in a 15% polyacrylamide gel. Samples of purified SLN1-R1 and YPD1 proteins were applied as molecular-weight standards.

3. Results and discussion

Recent advances in the field have indicated that phosphate analogs, particularly BeF_3^-, will bind non-covalently and activate response-regulator proteins (Cho *et al.*, 2001; Lee *et al.*, 2001; Yan *et al.*, 1999). Therefore, varying ratios of $BeCl_2$ and NaF were included with the YPD1 and SLN1-R1 protein mixture in order to help promote complex formation. Two different crystal forms were obtained at room temperature by the hanging-drop vapor-diffusion method using ammonium sulfate as a precipitant (Fig. 1). Crystals grew from 2.4–2.8 *M* ammonium sulfate at pH 5 or 6. Further optimization of the crystallization conditions produced diffraction-quality crystals using 2.4–2.6 *M* ammonium sulfate, 50 m*M* sodium acetate pH 5.3.

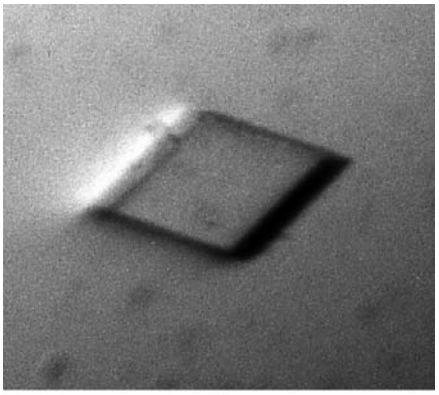
Preliminary X-ray analysis revealed that two different crystal forms grew under similar conditions. Crystal form I (Fig. 1*a*) belongs to a trigonal space group ($P3_2/P3_1$), with unit-cell parameters $a = b = 91.4$, $c = 201.1$ Å, whereas crystal form II (Fig. 1*b*) belongs to an orthorhombic space group ($P2_12_12_1$), with unit-cell parameters $a = 51.6$, $b = 74.2$, $c = 98.7$ Å. We even observed smaller trigonal crystals that form under the same crystallization conditions as the larger orthorhombic crystal, as shown in Fig. 1(*b*). The average dimensions of the crystals formed were 0.15 × 0.15 × 0.1 mm for both geometries. Although crystals were obtained both in the absence and presence of beryllium fluoride, a higher percentage of orthorhombic crystals were formed in the presence of the phosphate analog.

Table 1
Summary of X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Trigonal	Orthorhombic
Possible space group	$P3_1$ or $P3_2$	$P2_12_12_1$
Resolution range	30.0–2.10	30.0–2.30
Unique reflections	104137	17135
Mean $I/\sigma(I)$	30.7 (6.4)	19.4 (6.8)
R_{sym}† (%)	5.3 (18.6)	5.0 (23.9)
Completeness (%)	95.6 (72)	98.3 (94.5)
Multiplicity	5.13 (2.54)	4.9 (4.3)

† $R_{sym} = \sum(|I - \langle I\rangle|)/\sum I$, where I is the observed intensity and $\langle I\rangle$ is the mean intensity of the symmetry-equivalent reflection.



(*a*)

(*b*)

Figure 1
(*a*) Form I (trigonal) crystal of YPD1/SLN1-R1. Average crystal dimensions were ~0.15 × 0.15 × 0.05 mm. (*b*) A single large form II (orthorhombic) crystal of YPD1/SLN1-R1 surrounded by a number of smaller trigonal crystals growing in the same hanging drop. Average crystal dimensions for orthorhombic crystals were ~0.15 × 0.15 × 0.1 mm.

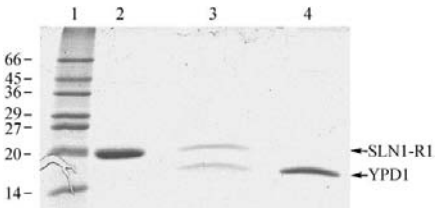


Figure 2
SDS–PAGE analysis of the YPD1/SLN1-R1 orthorhombic crystals. Lane 1, molecular-weight markers (kDa); lane 2, purified YPD1 (∼20 µg); lane 3, dissolved YPD1/SLN1-R1 crystals; lane 4, purified SLN1-R1 (∼10 µg). Proteins were denatured in SDS–PAGE sample buffer, separated on a 15% polyacrylamide gel and stained with Coomassie blue.

X-ray data were collected from form I and form II crystals grown in the presence of beryllium fluoride. Complete data sets to 2.1 and 2.3 Å were obtained at 100 K for crystal form I and II, respectively, at the University of Oklahoma protein X-ray facility. For crystal form I (trigonal), high- and low-resolution data sets were collected from the same crystal and subsequently merged to give 95.6% completeness overall. Table 1 summarizes the X-ray data-collection statistics for both crystal forms.

To assess whether the crystals contained SLN1-R1 alone, YPD1 alone or a YPD1–SLN1-R1 complex, crystals of each form were rinsed and dissolved in SDS–PAGE loading buffer and their content was analyzed by SDS–PAGE. As shown in Fig. 2 (lane 3), analysis of the orthorhombic crystals revealed a 1:1 stoichiometric ratio of YPD1 and SLN1-R1 protein. Similar analysis of the trigonal crystals confirmed that they too contained a 1:1 ratio of both proteins (data not shown). However, it is not yet known whether the proteins pack in these crystals independently of one another or whether they are in the form of a complex.

The calculated Matthews coefficient (Matthews, 1968) is 2.33 $Å^3\ Da^{-1}$ (solvent content of ∼44%) for the form I crystals (trigonal), assuming there to be six 1:1 YPD1–SLN1-R1 complexes in the asymmetric unit. In the case of the form II (orthorhombic) crystals, a Matthews coefficient of 2.74 $Å^3\ Da^{-1}$ (∼55% solvent) can be calculated assuming the presence of one 1:1 complex in the asymmetric unit.

Molecular-replacement methods will be employed for structure determination using the YPD1 model (PDB code 1qsp) as a search model. The structure of a YPD1–SLN1-R1 complex will provide valuable information regarding molecular recognition and interaction of HPt proteins with their cognate response-regulator proteins.

We thank Eric Enwall for assistance during data collection, Qingping Xu for help with data processing and members of the West laboratory for critical reading of the manuscript. This work was supported by a grant from the Oklahoma Center for the Advancement of Science and Technology (HR99-080) and a Public Health Service grant (GM59311) from the National Institutes of Health. AHW is a Cottrell Scholar of Research Corporation.

References

Cho, H., Wang, W., Kim, R., Yokota, H., Damo, S., Kim, S.-H., Wemmer, D., Kustu, S. & Yan, D. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 8525–8530.
Janiak-Spens, F., Sparling, J. M., Gurfinkel, M. & West, A. H. (1999). *J. Bacteriol.* **181**, 411–417.
Lee, S.-Y., Cho, H. S., Pelton, J. G., Yan, D., Henderson, R. K., King, D. S., Huang, L.-S., Kustu, S., Berry, E. A. & Wemmer, D. E. (2001). *Nature Struct. Biol.* **8**, 52–56.
Maeda, T., Wurgler-Murphy, S. M. & Saito, H. (1994). *Nature (London)*, **369**, 242–245.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Ota, I. M. & Varshavsky, A. (1993). *Science*, **262**, 566–569.
Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 56–62. Warrington: Daresbury Laboratory.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pflugrath, J. W. (1999). *Acta Cryst.* D**55**, 1718–1725.
Porter, S. W., Xu, Q. & West, A. H. (2003). *Eukaryot. Cell*, **2**, 27–33.
Posas, F., Wurgler-Murphy, S. M., Maeda, T., Witten, E. A., Thai, T. C. & Saito, H. (1996). *Cell*, **86**, 865–875.
Saito, H. (2001). *Chem. Rev.* **101**, 2497–2509.
Song, H. K., Lee, J. Y., Lee, M. G., Min, J. M. K., Yang, J. K. & Suh, S. W. (1999). *J. Mol. Biol.* **293**, 753–761.
Xu, Q., Nguyen, V. & West, A. H. (1999). *Acta Cryst.* D**55**, 291–293.
Xu, Q. & West, A. H. (1999). *J. Mol. Biol.* **292**, 1039–1050.
Yan, D., Cho, H. S., Hastings, C. A., Igo, M. M., Lee, S.-Y., Pelton, J. G., Stewart, V., Wemmer, D. E. & Kustu, S. (1999). *Proc. Natl Acad. Sci. USA*, **96**, 14789–14794.