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Crystallization and preliminary X-ray crystallographic studies of response regulator for cyanobacterial phytochrome, Rcp1

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The key response-regulator gene of light regulation, *rcp1*, from *Synechocystis* sp. has been overexpressed, purified and subsequently crystallized using ammonium sulfate as a precipitant in forms suitable for X-ray crystallographic studies. A native data set was collected to a resolution of 2.5 Å at cryogenic temperature. The crystals belong to the hexagonal space group $P6_3$, with unit-cell parameters $a = b = 89.04$ (5), $c = 60.29$ (3) Å. The Matthews parameter suggests that Rcp1 crystallizes with two molecules per asymmetric unit.

1. Introduction

Plants detect light using various photoreceptors such as phytochromes. The biliprotein phytochrome regulates plants' growth and developmental responses to the ambient light environment through an unknown mechanism (Yeh *et al.*, 1997). All plants, some algae and at least some cyanobacteria have phytochromes or phytochrome-like proteins (Reed, 1998). In cyanobateria, the initial step of light signal transduction by phytochrome is regulated by the phosphorelay, a complex signal transduction system consisting of kinases and response regulators. The key component of a phosphorelay is the phosphotransferase, which recognizes a response regulator and transfers a phosphoryl group between them (Tzeng *et al.*, 1998). The cyanobacterial phytochrome (Cph1) is a light-regulated histidine kinase that mediates red/infrared reversible phosphorylation of a small response regulator, Rcp1. The *cph1* sequence encodes a putative protein with an N-terminal chromophore domain resembling that of higher plant phytochromes (Reed, 1998). The C-terminal domain resembles a histidine-kinase domain of the bacterial two-component family. Its enzymatic activities are regulated in a similar way as has been recently demonstrated for the serine/threonine-kinase activities of plant phytochromes (Yeh & Lagarias, 1998; Fankhauser *et al.*, 1999). *rcp1* encodes a 147 amino-acid protein related to the CheY superfamily of bacterial response regulators, which contain aspartate-kinase receiver modules (Yeh *et al.*, 1997). The sensor kinase interprets specific signals resulting in an autophosphorylation reaction where a phosphate from ATP is transferred to a histidine residue on the kinase (Ninfa & Magasanik, 1986). The phosphoryl group is then donated to an aspartate residue on the response regulator. The phosphorylation of the response regulator serves to modify downstream signal transduction (Stock *et al.*, 1989; Parkinson & Kofoid, 1992). In infrared light or in darkness, Cph1 phosphorylates itself at a histidine residue and then transfers the phosphate group to the Rcp1 protein, which puts Rcp1 in a signalling state for regulating an unknown response. In red light, Rcp1 is not phosphorylated and exists in the opposite signalling state (Reed, 1998). In order to provide the structural basis of the light-response regulation by Cph1 and Rcp1, we have initiated the structure determination of Rcp1. As the first step toward its structural elucidation, we report the overexpression, purification and crystallization of Rcp1 and the preliminary X-ray crystallographic analysis.

2. Materials and methods

2.1. Protein purification

pTYB2 (New England BioLabs, Inc.) vector containing the *rcp1* gene (locus slr0474) (GB:D64001, locus 1001166) was transformed into *Escherichia coli* strain ER2566 (New England BioLabs, Inc.). Expression of Rcp1 protein tagged with intein-maltose binding protein was induced by 1 m*M* IPTG (isopropyl β-D-thiogalactopyranoside) at 293 K for 14–16 h. All the cultures were grown in modified Luria–Bertani medium (5 g yeast extract, 10 g tryptone, 5 g NaCl and 2 g dextrose per 1 l medium). Cultured cells suspended in lysis buffer (20 m*M* Tris–HCl pH 8.0, 0.5 m*M* EDTA) were disrupted by sonication. The cell lysate was centrifuged at 4000*g* to remove the pellet. The supernatant was applied to IMPACT (intein-mediated purification with an affinity chitin-binding tag, New England BioLabs, Inc.) affinity chromatography beads. After batch binding and washing, Rcp1 was cleaved from intein tag bound to chitin beads by incubating the beads with elution buffer

Table 1
Summary of the Rcp1 data-collection statistics.

Values in parentheses are for the highest resolution shell.

Data set	Native	TMLA derivative
X-ray source	Rigaku RU-300	PF (BL-6A)
Wavelength (Å)	1.54	1.00
Space group	$P6_3$	$P6_3$
Unit-cell parameters (Å)	$a = b = 89.04$, $c = 60.29$	$a = b = 89.09$, $c = 60.21$
Resolution (Å)	2.5	2.7
Total No. of reflections	31847	63692
No. of unique reflections	9553	7599
Completeness (%)	93.5 (99.7)	99.1 (100)
$R_{\rm merge}$† (%)	6.7 (34.6)	6.2 (29.3)
Mean $I/\sigma(I)$	17.8 (5.5)	13.6 (7.7)

† $R_{\rm merge}\ (I) = \sum_h \sum_i |I_i - I| / \sum_h \sum_i I$, where I is the mean intensity of i reflections h.

(20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 30 m*M* dithiothreitol) for 14 h. Eluate containing Rcp1 was further purified to near-homogeneity by gel filtration on a Superdex 75 column (Hiload 16/60, Pharmacia) previously equilibrated with storage buffer (20 m*M* HEPES–NaOH pH 7.5, 150 m*M* KCl) and was concentrated by ultrafiltration to 8 mg ml^{-1} (Centriprep 10, Amicon) for crystallization.

2.2. Crystallization

Rcp1 was crystallized at room temperature (294 ± 1 K) using the hanging-drop vapour-diffusion method. Crystals were grown on siliconized cover slips by equilibrating a mixture containing 8 µl of protein solution and 8 µl of reservoir solution (3.1–3.2 *M* ammonium sulfate, 0.1 *M* MES–NaOH pH 6.6) against 1 ml of reservoir solution. Protein solution was prepared by eightfold dilution of the concentrated protein solution (8 mg ml^{-1} protein in 20 m*M* HEPES–NaOH pH 7.5, 150 m*M* KCl) with distilled water.

2.3. Data collection and molecular-replacement calculations

For the cryogenic experiments, a suitable cryoprotectant was determined to be 2.4 *M* ammonium sulfate, 0.1 *M* MES–NaOH pH 6.6 plus 30%(*w*/*v*) sucrose. Successful flash-freezing was achieved when the crystals were transferred directly from the drop to cryosolvent containing 30% sucrose and were allowed to equilibrate for 2 min. A heavy-atom-containing derivative search was performed by soaking the native crystals in appropriate solutions. The first set of native data was collected on an R-AXIS IV image-plate system attached to a Rigaku rotating-anode generator (RU-300) providing Cu $K\alpha$ radiation and running at 50 kV and 90 mA with a 0.3 mm focus cup in a nitrogen-gas stream at 110 K (Oxford Cryosystems). For heavy-atom derivatization, crystals were soaked in reservoir solution containing 10 m*M* trimethyllead acetate (TMLA) for 24 h. Diffraction data from the TMLA derivative were collected at the Photon Factory in Japan, using the X-ray beam BL6A (1.00 Å with an Si monochromator system) from a 2.5 GeV synchrotron-radiation source with a Weissenberg camera for protein crystallography (Sakabe, 1991). The imaging plates were digitized at 100 µm intervals with a Fujix BAS2000 read-out system (Fuji Photo Film Co., Ltd). Data sets were indexed and processed with *DENZO* (Otwinowski & Minor, 1997) and scaled and merged with *SCALEPACK* (Otwinowski & Minor, 1997). Molecular-replacement calculations were carried out with *AMoRe* (Navaza, 1994) using starting models based on CheY (PDB code 1chn; Volz & Matsumura, 1991), NarL (PDB code 1rnl; Baikalov *et al.*, 1996), Spo0F (PDB code 1srr; Madhusudan *et al.*, 1996) and PhoB receiver domains (PDB code 1b00; Sola *et al.*, 1999). Sequence alignment of Rcp1 and other response regulators used in MR is shown in Fig. 1.

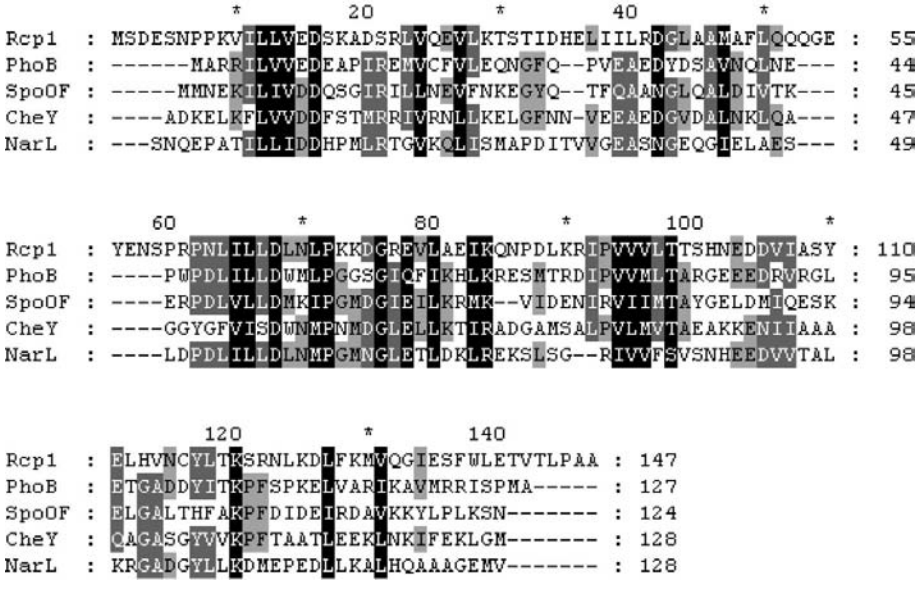

Figure 1
Sequence alignment of Rcp1 and other response regulators. Amino-acid sequences of *Synechocystis* sp. Rcp1, *E. coli* PhoB receiver domain, *Bacillus subtilis* Spo0F, *E. coli* CheY and *E. coli* NarL receiver domain were aligned using *ClustalX*. Highly conserved residues are shaded black and similar residues are shaded grey. Sequence similarities between Rcp1 and PhoB, Spo0F, CheY and NarL are 41, 40, 42 and 45%, respectively.

3. Results and discussion

Protein purification was carried out using the IMPACT affinity system and a Superdex 75 column. After the initial affinity chromatography, protein was sufficiently pure for crystallization and Superdex 75 gel filtration was subsequently applied to exchange the buffer. From the crystallization preparation described above, microcrystals began to appear in two weeks and grew into hexagonal crystals with typical dimensions 0.12 × 0.12 × 0.12 mm in two to four months (Fig. 2). Rcp1 was crystallized using a very high salt concentration (3.2 *M* ammonium sulfate) as a precipitant. This crystallization property of Rcp1 restricted the number of precipitants available for substituting the ammonium sulfate and the use of other additives. The slow growth rate of the Rcp1 crystal made it difficult to optimize the crystallization conditions and the heavy-atom derivative search. The native data set was collected using Cu $K\alpha$ X-rays and the R-AXIS IV system. Diffraction patterns from flash-frozen Rcp1 crystals were observed to a limit of 2.5 Å on the image plate of the detector. The statistics of the native data set and one heavy-atom derivative data are summarized in Table 1. In the highest resolution shell of the native data, the data was 99.7% complete and $I/\sigma(I) = 5.5$. The data set consisted of a total of 31 847 measurements of 9553 unique reflections ($R_{\rm merge}$ is 6.7%). Autoindexing with *DENZO* indicated a hexagonal space group with unit-cell parameters $a = b = 89.04$ (5), $c = 60.29$ (3) Å. Based on the results from *SCALEPACK* and from the Matthews parameter V_m, it is most likely that the space group is $P6_3$ and there are two molecules in

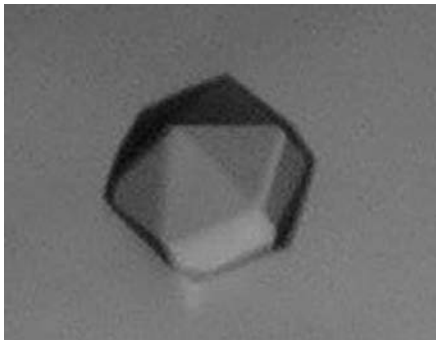

Figure 2
A hexagonal crystal of Rcp1 grown for two months using 3.2 *M* ammonium sulfate as a precipitating agent (pH 6.6). Its approximate dimensions are 0.1 × 0.1 × 0.1 mm.

asymmetric unit. The unit-cell volume is 413 950 Å^3 and the molecular weight of each subunit (147 amino acids) is 16 572 Da. Assuming that there are two molecules in the asymmetric unit, V_m of these crystals is 2.08 Å^3 Da^{-1} and the solvent content is 40.9%; these values are well within the range of previously observed protein crystals (Matthews, 1968). Initially, the molecular-replacement method was tried extensively using the three-dimensional structures that have high sequence homologies with Rcp1, but no distinct solution appeared. For multiple isomorphous replacement, various heavy atoms were tested and the TMLA derivative was found to be helpful for partial phasing. The crystal structure determination of Rcp1 by a combination of multiple iosmorphous replacement and multi-wavelength anomalous dispersion methods is now under way.

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