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Crystallization and preliminary X-ray diffraction studies on the DNA-binding domain of the transcriptional activator protein PhoB from *Escherichia coli*

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PhoB is a transcriptional factor that activates more than 30 genes of the *pho* regulon in response to phosphate starvation. Crystals of its C-terminal domain (PhoBC) were obtained in two forms. The first crystal form, obtained from phosphate solution, belongs to space group $P2_1$, with unit-cell parameters a = 30.7, b = 105.9, c = 30.9 Å, β = 110.3°. The second form, crystallized from PEG solution, belongs to the same space group, but has a smaller unit cell (a = 30.6, b = 37.5, c = 44.4 Å, β = 109.4°). Crystals of selenomethionyl-derivatized PhoBC were obtained using the conditions for the second crystal form. Diffraction data from wild-type PhoBC (2.0 Å resolution) and MAD data sets from selenomethionyl-derivative PhoBC (3.0 Å resolution) have been collected at 100 K with a synchrotron-radiation source. MAD data analysis is in progress.

1. Introduction

'Two-component' signal transduction pathways are remarkably common in eubacterial responses to changes in the local environment (Tae-Yoon *et al.*, 1989) and have been identified in eukaryotic cells (Chang *et al.*, 1993). Such two-component pathways are involved in a wide array of processes including phosphate regulation, nitrogen regulation and osmoregulation.

In inorganic phosphate regulation, this system is controlled by the transmembrane sensor kinase PhoR and the response regulator PhoB, the latter being activated by the transfer of a phosphate group from the former (Makino *et al.*, 1989). PhoB is a transcription activator that, once activated, induces the expression of genes belonging to the phosphate (*pho*) regulon (Shinagawa *et al.*, 1983). PhoB (229 amino-acid residues) consists of two distinct functional and structural domains: an N-terminal receiver domain (120 residues) that is phosphorylated (Hiratsu *et al.*, 1995) and a C-terminal output domain (PhoBC, 104 residues) that interacts with *pho* promoters (Makino *et al.*, 1996) and also with the σ^{70} subunit of RNA polymerase (Pao & Saier, 1995). Like the related OmpR protein (Martinez-Hackert & Stock, 1997; Kondo *et al.*, 1997), PhoBC contains a helix–turn–helix motif, as observed in the recently determined NMR structure (Okamura *et al.*, 2000).

All *pho* regulon genes, including *phoB*, are preceded by a promoter that contains an upstream activation site in place of the −35 sequence termed the *pho* box. The *pho* box is composed of two 7 bp direct repeats with a conserved consensus sequence CTGTCAT separated by a 4 bp AT-rich spacer region (Makino *et al.*, 1986). Mutant studies have identified the residues involved in DNA binding and in the interaction with the σ^{70} subunit of RNA polymerase (Makino *et al.*, 1996). Some of these essential residues (*e.g.* Gly185 and Val190 in the long loop between helices 2 and 3) are poorly defined in the structure of PhoBC determined by NMR spectroscopy (Okamura *et al.*, 2000).

As an initial step to further clarify the molecular binding of PhoBC to the *pho*-box DNA and its interaction with the σ^{70} subunit of RNA polymerase, we have attempted to undertake a crystallographic study of PhoBC and here describe the crystallization and preliminary X-ray diffraction analysis of PhoBC.

2. Expression and purification

Expression and purification of PhoBC were performed essentially as described in Makino *et al.* (1996), with some modifications. The overexpression plasmid pTBC104 was constructed for PhoBC (the C-terminal residues 126–229 of PhoB) by placing the coding region under the T7 promoter of plasmid pT7-7. *Escherichia coli* cells [BL21(λDE3) strain] were grown at 310 K in LB broth and IPTG was added (to a final concentration of 1 m*M*) at A_{600} = 0.8. After 4 h, the cells were harvested. The cell extract prepared by sonication was subjected to a 50% saturation ammonium sulfate cut. A three-step column chromatography protocol was adopted: the

first step used a CM-Toyopearl column (TOSOH) or a cellulose phosphate (Whatman, P11) column, the second a MonoS column (Pharmacia) and the final step a Superdex75 column (Pharmacia). The purified protein was concentrated to 15–20 mg ml^{-1}. 1 l of culture yielded approximately 7 mg of pure protein. A chimeric PhoBC molecule, comprising the authentic PhoBC and seven residues derived from an overexpression vector, was expressed and purified in the same way as for the authentic PhoBC and was used in the initial stages of the crystallization study.

In the selenomethionine labelling of PhoBC, the *E. coli met*$^-$ auxotropic strain B834(DE3) (Novagen) was used as a host for plasmid transformation. Cells were grown at 310 K in M9 media including 25 mg l^{-1} of selenomethionine and IPTG was added (to a final concentration of 1 m*M*) at A_{600} = 1.0. After 15 h, the cells were harvested. The selenomethionyl (Se-Met) PhoBC was purified by an identical procedure to that for the wild-type PhoBC, except that it was purified under strictly reducing conditions. 1 l of culture yielded approximately 1.5 mg of purified protein.

3. Crystallization

Crystallization conditions were screened using the microbatch method. For the first crystal form (crystal form I), an extensive search for crystallization conditions was conducted using chimeric PhoBC with a small-scale batch method, in the same way as described for thermophilic F_1-ATPase (Shirakihara *et al.*, 1991). The subsequent refinement gave the best crystallization conditions, which employed the micro-dialysis method with 3 ml exterior solution consisting of 1.8–2.0 *M* sodium/potassium phosphate, 50 m*M* MES pH 5.7 and with 25 µl interior solution containing 1.45 *M* sodium/potassium phosphate, 50 m*M* MES pH 5.7 and 8 mg ml^{-1} protein. Under these conditions, rectangular parallelepiped-shaped crystals of dimensions 0.5 × 0.5 × 0.2 mm appeared after two months. The chimeric PhoBC crystals exhibited high mosaicity and were therefore of no further use. However, the conditions were found to be applicable to the authentic PhoBC. The first crystal form required higher purity for successful crystallization than the second crystal form (described below).

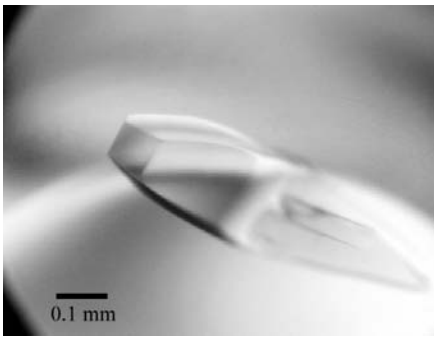

Figure 1
Crystals of Se-Met PhoBC. Crystals were grown at 298 K from a crystallization mixture containing 15% PEG 8000, 0.1 *M* sodium acetate, 250 m*M* NaCl, 50 m*M* sodium cacodylate pH 6.8 and 10 mg ml^{-1} protein.

For the second crystal form, a similar search was conducted at 298 K using commercial sparse-matrix screening kits (Hampton Research). Typically, 1 µl protein solution (20 mg ml^{-1} PhoBC, 20 m*M* Tris–HCl pH 8.0 and 0.5 *M* NaCl) was mixed in a 1:1 ratio with each of the crystallization solutions and the resultant 2 µl drop was incubated at 293 K. In this search, the crystallization conditions for the second crystal form were found, along with conditions equivalent to those for the first crystal form. Subsequent optimization refined the second conditions to the batch method with 2 µl of a mixture consisting of 17% PEG 8000, 50 m*M* sodium cacodylate, 100 m*M* sodium acetate pH 6.8, 250 m*M* NaCl, 10 mg ml^{-1} protein. Under these conditions, rectangular parallelepiped-shaped crystals appeared within 7 d and reached maximum dimensions of 0.10 × 0.10 × 0.20 mm after two weeks, although these crystals were not very reproducible. From a systematic search for a cryosolution, the above-mentioned reservoir solution containing 20% glycerol was found to be suitable.

For the second crystal form, crystals of selenomethionyl-PhoBC were obtained under the almost same conditions as those for the wild-type PhoBC, except with a slightly lower PEG concentration (15%) (Fig. 1). The cryoprotection conditions were also almost the same as those for the wild-type PhoBC.

4. X-ray diffraction

For the crystal form I obtained from authentic PhoBC, diffraction data were collected from a crystal mounted in a capillary using synchrotron radiation and the Weissenberg camera installed at beamline BL18B of the Photon Factory, KEK, Tsukuba. Crystals diffracted to 2.0 Å resolution and showed good stability towards X-ray irradiation at ambient temperature. The crystals belong to space group $P2_1$, with unit-cell parameters a = 30.6, b = 105.9, c = 30.9 Å, β = 110.3°. The asymmetric unit contains two molecules of PhoBC (40.8% solvent content). The data were integrated with *DENZO* (Otwinowski & Minor, 1997) and reduced with *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) with $R_{\rm merge}$ = 10.4% (2.0 Å resolution, 68% data completeness).

For the wild-type crystal form II, crystals were flash-frozen at 100 K in a nitrogen cryostream after being immersed in the cryosolution for 5–10 s. Intensity data were collected at 100 K on beamline BL40B2 at SPring-8 (Harima, Japan) using a CCD detector and 1.0 Å wavelength X-ray radiation. The diffraction data were integrated using *MOSFLM* (Leslie, 1992) and reduced using *SCALA* with $R_{\rm merge}$ = 6% (2.0 Å resolution, 99.0% data completeness). The crystals belong to space group $P2_1$, with unit-cell parameters a = 30.6, b = 37.5, c = 44.4 Å, β = 109.4°. The asymmetric unit contains one PhoBC molecule (38.6% solvent content). MAD intensity data sets to 3.0 Å resolution have been collected at three different wavelengths on beamline BL40B2 at SPring8 [$R_{\rm merge}$ and completeness of 4.1 and 99.1%, respectively, for wavelength 0.9801 Å (edge), of 4.6 and 99.3%, respectively, for wavelength 0.9798 Å (peak) and of 4.5 and 99.6%, respectively, for 0.9879 Å (remote)]. Patterson map analysis of MAD data using *SOLVE* (Terwilliger & Berendzen, 1999) located all of the five possible selenium sites. Initial experimental electron-density maps show clear protein density fitting well to the NMR structure; structure determination is in progress.

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