Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallographic characterization of a novel protein SixA which exhibits phospho-histidine phosphatase activity in the multistep His–Asp phosphorelay

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Received 23 February 1998
Accepted 1 June 1998

SixA has been isolated from *Escherichia coli* as the first protein to exhibit phospho-histidine phosphatase activity. Recent biochemical studies have shown that SixA is involved in the signal transduction of the His–Asp phosphorelay through the dephosphorylation of the histidine-containing phosphotransfer (HPt) domain of the anaerobic sensor kinase ArcB. Crystals of SixA were obtained using a hanging-drop vapour-diffusion method with polyethylene glycol and calcium ions. Preliminary X-ray crystallographic analysis revealed that the crystals belonged to space group $P2_12_12_1$ with unit-cell dimensions a = 39.26, b = 48.62 and c = 83.18 Å, having one molecule in the crystallographic asymmetric unit. The intensity data were collected up to 1.5 Å resolution using synchrotron radiation.

1. Introduction

In bacterial cells, the two-component system of intracellular signal transduction is widely involved in the regulation of gene expression and cell behaviour. Recent studies have shown that yet another variation of the two-component system exists in yeasts (Ota & Varshavsky, 1993; Maeda *et al.*, 1994), in fungi (Alex *et al.*, 1996) and in plants (Chang *et al.*, 1993). The sensor and regulator proteins contain common characteristic modules: a transmitter and a receiver, respectively (Stock *et al.*, 1990; Parkinson & Kofoid, 1992; Alex & Simon, 1994). Upon stimulation, a conserved histidine residue of the transmitter module is autophosphorylated in an ATP-dependent manner. Then, the phosphoryl group bound to the histidine residue is transferred to a conserved aspartic acid residue in the receiver module of the response regulator. For this reason, the system is also known as the His–Asp phosphorelay. Recent biochemical studies have shown that multistep phosphorelaying occurs in several signalling systems (Appleby *et al.*, 1996; Wurgler-Murphy & Saito, 1997). One of the most extensively studied multistep phosphorelays is the signalling system ArcB–ArcA (Ishige *et al.*, 1994; Tsuzuki *et al.*, 1995), which anaerobically represses the expression of operons which encode the enzymes that are involved in aerobic metabolism (Lin & Iuchi, 1991). The sensor kinase ArcB contains two transmitter modules and one receiver module. Upon stimulation, the histidine residue of the first transmitter module is autophosphorylated and transfers the phosphoryl group to the aspartic acid residue of the receiver domain. The receiver domain then transfers the phosphoryl group to the histidine residue of the second transmitter module, which is referred to as the HPt (histidine-containing phosphotransfer) domain. Finally, the HPt domain transfers the phosphoryl group to the aspartic acid residue of the receiver domain of the response regulator ArcA, which is a transcription factor.

Although the physiological meaning of the multistepping in this phosphorelay is not fully understood, it has been pointed out that one of the most important advantages of the multistep model may be its potential for multiple regulatory checkpoints (Grosmann, 1995; Posas *et al.*, 1996). Phosphatases are the most preferred candidates for regulating the signal flow in phosphorelaying (Perego *et al.*, 1994; Hess *et al.*, 1988). Using *in vivo* screening, SixA has been isolated from *E. coli* as the first protein to exert phospho-histidine phosphatase activity and as the first to exhibit a phosphatase activity specific for the phospho-histidine of the HPt domain of ArcB (Ogino *et al.*, 1998). The protein consists of 161 residues (17.2 kDa) and has an arginine–histidine–glycine (RHG) signature at the N-terminus. This RHG motif, which presumably functions as a nucleophilic phosphoacceptor, was reported for several phosphatases, including *E. coli* periplasmic phosphatase and glucose-1-phosphate phosphatase, eukaryotic fructose-2,6-bisphosphatase and acid phosphatase and ubiquitous phosphoglycerate mutase. The entire amino-acid sequence of SixA, however, does not resemble any of these enzymes. Using predicted amino-acid sequences on the entire genomic databases, two putative homologous proteins have been found in *Haemophilus influenzae* Rd and *Synechocystis* sp PCC 6803, both of which have hybrid sensor kinases containing a HPt domain, although SixA has relatively low identities with these proteins, the sequence identities being 21 and 38%, respec-

tively. It has been demonstrated that the purified SixA exhibits dephosphorylation activity against the HPt domain of ArcB, but the mutant protein lacking the histidine residue in the RHG motif does not. Evidence has also been provided that a deletion mutation of the *sixA* gene on the chromosome affects the *in vivo* phosphotransfer signalling.

We have been focusing our attention on the structural biology of the multistep phosphorelay of the ArcB–ArcA pathway and trying to crystallize the proteins or their domains. Recently, we crystallized and solved the crystal structure of the HPt domain of ArcB to reveal the structure–function relationship (Kato *et al.*, 1996, 1997). In this paper, we report the purification and physicochemical and crystallographic characterization of SixA.

2. Materials and methods

Construction of the *E. coli* strain and plasmids used in this study have been described previously (Ogino *et al.*, 1998). SixA was overproduced in *E. coli* BL21(DE3) cells harbouring plasmid pET-RX6, which carries a gene encoding SixA under the T7 promoter. The protein was purified by an ammonium sulfate fractionation (precipitant at 45% saturated ammonium sulfate) and three steps of column chromatography, using DEAE-Sepharose FF, Mono-Q and Sephacryl S-100 HR (all from Pharmacia Biotech, Uppsala, Sweden). The purified protein was concentrated at 20 mg ml^{-1} using an ultrafiltration membrane (AMICON Centriplus3, Beverly, Massachusetts, USA). In this purification scheme, 30 mg of purified protein was obtained from 20 g of wet cells. The concentration was determined using the absorption measurement with the calculated molar absorption coefficient (ε_{280} = 13940) from the sum of the ε_{280} values of two tryptophan and two tyrosine residues of SixA. The resulting sample was verified with matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) (JMS-ELITE, PerSeptive Inc, Framingham, Massachusetts, USA), and N-terminal analysis (The Procize, Applied Biosystems, Foster City, California, USA). The observed molecular weight (17203) was virtually identical to the calculated value (17207). Circular dichroism (CD) of the protein was measured with a JASCO 720W spectrometer (Japan). CD spectra showed that the protein structure was stable at pH values between 5.0 and 9.0. An estimation of the composition of secondary-structure elements from the CD spectra at pH 6.6 using a reference CD method (Yang *et al.*, 1986) indicated that the protein contains a high amount of β-strand (45%) with 16% α-helix. Gel filtration of the protein was carried out using a Superose 12 (Pharmacia-LKB) equilibrated with a solution containing 50 m*M* Tris–HCl (pH 7.8), 0.1 *M* NaCl, 1 m*M* DTT and 1 m*M* EDTA. The elution profile contained two peaks corresponding to 44 kDa and 33 kDa, which were calculated from a calibration curve. The ratio of the peak at 44 kDa to the peak at 33 kDa was 1:4.

The screening of the crystallization conditions of SixA was performed by means of a hanging-drop vapour-diffusion technique using standard crystallization solutions (Hampton Research), followed by refinement of the conditions by changing the buffer solutions, precipitants and temperature or by adding metal ions and detergents.

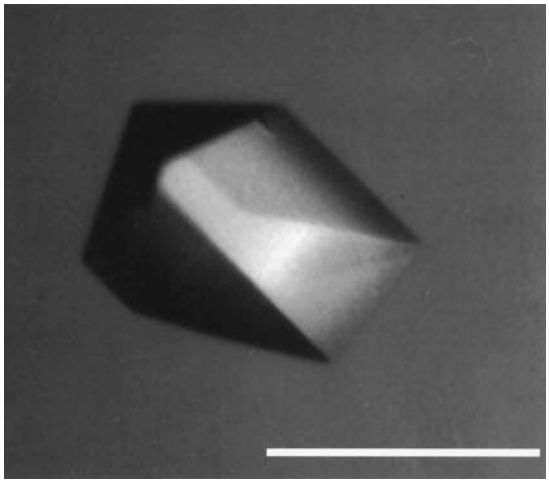

Figure 1
Crystal of SixA. The scale bar is 0.3 mm long.

X-ray diffraction data were collected at 283 K with a Rigaku imaging-plate area detector (R-AXIS IIc) using Cu $K\alpha$ radiation. Intensity data were processed with the program *PROCESS* (Rigaku, Japan). High-resolution data were collected at 288 K using synchrotron radiation (1.00 Å) and a Weissenberg camera (Sakabe, 1991) installed on beamline 18B at the Photon Factory (Tsukuba, Japan). Intensity data were processed with the program *DENZO* (Otwinowski, 1993). One crystal was used for each data collection in the laboratory and at the Photon Factory.

3. Results and discussion

Needle-like crystals (form I) of SixA were first obtained from a solution containing 100 m*M* Mes-K buffer (pH 6.6), 250 m*M* LiCl and 10% polyethylene glycol (PEG) 6K. The crystals tended to form clusters, which made it difficult to isolate a single crystal for the diffraction experiments. The crystals belong to space group *C*2 with unit-cell parameters a = 115.3, b = 49.3, c = 69.7 Å, β = 105.3°, and were estimated to contain two molecules in the asymmetric unit with a V_m value of 2.5 Å^3 Da^{-1}. This may have been a factor in the predominance of the dimer form in solu-tion as indicated by gel filtration. In the X-ray experiments, the decay of the crystals was rather rapid, although they diffracted to 2.2 Å resolution. Similar crystals were also obtained from a solution containing 100 m*M* Mes-K buffer (pH 6.0) and 20% PEG 6K and from a solution containing HEPES buffer (pH 7.0) and 0.8–1.0 *M* ammonium sulfate. Several attempts to minimize the problems described above with additives including dioxane, glycerol or glucose were unsuccessful.

Chunky crystals (form II, Fig. 1) were found to form from solutions containing $CaCl_2$. The best crystals were obtained by vapour diffusion of a solution containing 50 m*M* Mes-K buffer (pH 6.6) with 10 mg ml^{-1} protein, 20 m*M* $CaCl_2$, 50 m*M* NaCl, 0.5 m*M* dithiothreitol (DTT) and 6% PEG 6 K using a reservoir containing 100 m*M* Mes-K buffer (pH 6.6), 40 m*M* $CaCl_2$, 50 m*M* NaCl and 12% PEG 6 K at 283 K. Typical dimensions were 0.3 × 0.2 × 0.1 mm. The crystals belong to space group $P2_12_12_1$ with a = 39.26, b = 48.62 and c = 83.18 Å. These crystals were estimated to contain one molecule in the asymmetric unit with a V_{solv} value of 0.41 and were found to diffract to at least 2.0 Å resolution. Similar crystals grew in the pH range 5.6–6.9.

X-ray diffraction data of form II crystals were collected at 2.06 Å resolution using an R-AXIS IIc detector with an angular range of 120° and a step size of 2° for an exposure time of 20 min. The resulting data gave an R_{merge} of 5.4% with a completeness of 98.9% (99.1% for the outer shell from 2.25 to 2.06 Å). The redundancy of reflections was 6.53. The total decay during data collection was 14% and the crystal mosaicity was 0.3. Diffraction data at 1.50 Å resolution were collected at the Photon Factory with an angular range of 119.1° and a step size of 3° for an exposure time of 18 s. Each frame overlapped with the preceding frame by 0.3°. The obtained data gave an R_{merge} of

6.8% with a completeness of 97.3% (95.6% for the outer shell from 1.55 to 1.50 Å) and a redundancy of 5.04. The crystal mosaicity was 0.48°. No decay during the data collection was detected. Structure analysis of these crystals using the multiple isomorphous replacement method is in progress.

This work was supported by Grants in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan to TM (06276105) and TH (06276104, 09235220, 09308025). MK was supported by a research fellowship from the Japan Society for the Promotion of Science.

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