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Characterization and crystallization of DivK, an essential response regulator for cell division and differentiation in *Caulobacter crescentus*

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DivK is an essential response regulator involved in the complex signal transduction network required for cell division and cell differentiation in *Caulobacter crescentus*. Small-angle X-ray scattering analysis was valuable for obtaining single crystals of the DivK recombinant protein. These crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 37.2, b = 40.5, c = 67.1 Å and diffract beyond 1.6 Å on a synchrotron beamline.

1. Introduction

In the Gram-negative aquatic bacterium *Caulobacter crescentus*, two-component signal transduction proteins play a crucial role in coordinating cell differentiation with cell-cycle progression (Hung *et al.*, 2000; Ohta *et al.*, 2000). This bacteria divides asymmetrically from the mother stalked cell into a new stalked cell and a new motile swarmer cell. The stalked cell initiates chromosome replication immediately, whereas the motile swarmer cell must first differentiate into a stalked cell before initiating DNA synthesis and entering the cell-division cycle (Newton & Ohta, 1990). Several studies revealed that the control of the different genetic programs in the two progeny cells involve histidine kinases and response regulators organized in a complex network of His-Asp phosphorelay cascades (Ohta *et al.*, 2000). It has also been shown that morphogenesis and cell-cycle progression is accompanied by the dynamic localization of the protein components of these cascades (Jacobs *et al.*, 2001; Wheeler & Shapiro, 1999).

The two histidine kinases PleC and DivJ, along with the response regulator DivK, act in a phosphorelay pathway to control activation of the global response regulator CtrA (Wu *et al.*, 1998). PleC accumulates at the flagellar pole of the swarmer cell and at the pole opposite to the stalk in predivisional cells. DivJ, in contrast, localizes to the base of the stalk at the time of stalk formation and remains at that pole throughout the cell cycle (Wheeler & Shapiro, 1999). DivK has been shown to be essential for cell viability (Hecht *et al.*, 1995). It is a single-domain protein and belongs to the subfamily of response regulators that also includes the Spo0F (Madhusudan *et al.*, 1996) and CheY (Stock *et al.*, 1989) proteins of enteric bacteria. Although the fold of these domains has been established from X-ray and NMR studies for a few members of this protein family (Foussard *et al.*, 2001; West & Stock, 2001), studies of the precise interactions between proteins for signal propagation in phosphorelay cascades has only been initiated in the *Bacillus subtilis* phosphorelay that controls sporulation (Hoch & Varughese, 2001). The essential involvement of DivK in a network of cell-cycle regulated phosphorelay cascades and the multiplicity of protein partners with which DivK establishes protein–protein interactions motivated the structural investigation on this protein.

2. Material and methods

2.1. Expression and purification

The *C. crescentus divK* gene was inserted into plasmid pT7-7 at the *Nde*I and *Bam*HI sites to generate plasmid pZHF55 in which the unaltered *divK* gene is under the control of the T7 promoter. The *Escherichia coli* strain BL21(DE3)pLysS, which carried plasmid pZHF55, was grown to an OD of 0.6 at 310 K in LB supplemented with ampicillin (100 µg ml^{-1}) and chloramphenicol (25 µg ml^{-1}). Expression of DivK was induced by addition of 1 m*M* IPTG for 2 h. The cells were harvested by centrifugation at 4000 rev min^{-1} for 30 min and washed in 50 m*M* Tris–HCl pH 7.0 buffer. All protein-purification procedures were carried out at 277 K and in the presence of 5 m*M* dithiothreitol (DTT). The bacterial pellet from a 2 l culture were suspended in ice-cold lysis-buffer (50 m*M* Tris–HCl pH 7.0, 50 m*M* KCl, 1 m*M* EDTA, 5 m*M* DTT, 0.1% Triton X-100, 10 µg ml^{-1} pepstatin, 5 µg ml^{-1} leupeptin, 1 m*M* PMSF, 2 µg ml^{-1} aprotinin and 10 µl benzonase 10000UI) and lysed by sonication.

Ammonium sulfate was added to the supernatant after centrifugation at 10 000 rev min^{-1} for 2 h. The protein fraction that precipitated between 1.2 and 1.4 *M* ammonium sulfate was dissolved in 20 m*M* Tris–HCl pH 7.0, 0.1 m*M* EDTA, 5% glycerol, 5 m*M* DTT (buffer *A*), centrifuged and dialyzed overnight against buffer *A*. The protein solution was loaded onto a 16/20 DEAE Sepharose column (Pharmacia) equilibrated in buffer *A* supplemented with 50 m*M* NaCl and eluted with a 100–200 m*M* NaCl linear gradient in buffer *A*. The DivK-containing fractions were concentrated on a Centriprep-10 (Amicon) and loaded onto a Superdex S75 16/100 prep-grade column (Pharmacia) equilibrated in buffer *A* supplemented with 100 m*M* NaCl. Pure DivK (125 amino acids) eluted as a single peak according to SDS–PAGE (silver stained). The protein concentration was determined spectrometrically at 280 nm and by the Bradford protein assay using bovine serum albumin as a standard.

2.2. Small-angle X-ray scattering measurements

The DivK protein was concentrated to 5 mg ml^{-1} in 20 m*M* MES–NaOH pH 6.0 and 20 m*M* Tris–HCl pH 7.0 or 8.0. The samples contained 20 m*M* DTT to eliminate the free radicals formed in solution under X-ray irradiation and were centrifuged for 1 h at 277 K and 15 000 rev min^{-1} to discard any insoluble material. X-ray scattering data were recorded on the small-angle scattering instrument D24 (Depautex *et al.*, 1987) using synchrotron radiation at the LURE–DCI storage ring (Orsay, France). The instrument and the data-acquisition system have been described previously (Bordas *et al.*, 1980). The wavelength of the X-rays was 1.488 Å (*K* edge of Ni) and the sample-to-detector distance was set at 1000 mm. Scattered intensities were recorded in the angular range $0.28 \times 10^{-2} < s$ (Å^{-1}) $< 6.43 \times 10^{-2}$, where s is the scattering vector, $s = (2\sin\theta)/\lambda$, and 2θ and λ are the scattering angle and wavelength of the X-rays, respectively. All experiments were performed at 277 K using a temperature-controlled cell (Dubuisson *et al.*, 1997). Eight successive frames of 200 s each were collected for each sample. The scattering intensity of a reference sample of carbon black, recorded immediately before and after data collection for each protein sample, was used to normalize all data to the transmitted intensity. The scattering contribution of the buffer was substracted before data analysis. The radius of gyration R_g was derived from Guinier's law (Guinier & Fournet, 1955),

$$I(s) = I(0)\exp(-4\pi^2 R g^2 s^2/3),$$

where $I(s)$ is the scattered intensity and $I(0)$ is the value of the extrapolated intensity at $s = 0$. For monodisperse solutions, the Guinier plot of $\ln[I(s)]$ *versus* s^2 approximates a straight line with a slope proportional to R_g in the small-angle region. After normalization to the protein concentration (c), the value of $I(0)/c$ is proportional to the molecular weight of the scattering species. The scattering data of a monodispersed solution of lysozyme of known concentration were used to calibrate this value.

2.3. Crystallization and data collection

Crystallization was performed by vapour equilibrium using the hanging- or sitting-drop method at 285 K. The protein was concentrated to 2 mg ml^{-1} in 20 m*M* MES–NaOH pH 6.0, 5 m*M* DTT and was mixed with an equal volume of the reservoir solution containing 32% polyethylene glycol monomethylether (PEG-MME) 550, 40 m*M* MES pH 6.0, 5 m*M* DTT, 0.01% NaN_3. Small needles appeared after 3 d in hanging drops. Crystal growth was significantly increased ($300 \times 40 \times 40$ µm) in 20 µl sitting drops. Crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 37.2$, $b = 40.5$, $c = 67.1$ Å. The calculation of the most likely Matthews parameters (Matthews, 1968) yields a value of 1.8 Å^3 Da^{-1} for one molecule per asymmetric unit and a solvent content of about 31%. For data collection, crystals were frozen in liquid propane after soaking for a few seconds in 50% PEG-MME 550, 40 m*M* MES pH 6.0. The vials were stored in liquid nitrogen. A native data set at 1.6 Å resolution was collected (low- and high-resolution data were measured independently) at 100 K on synchrotron beamline ID14-EH3 at the ESRF (Grenoble, France). The crystal mosaicity was 0.2°. Data processing and scaling were performed using *MOSFLM* (Leslie, 1987) and *SCALA* (Collaborative Computational Project, Number 4, 1994). Statistical parameters are given in Table 1.

3. Results and discussion

The DivK protein was produced and purified to the level of about 10 mg per litre of culture. The molecular mass of the recombinant protein from electrospray ionization mass spectrometry (14 041 Da) corresponded to the theoretical value minus the N-terminal methionine. The protein was monomeric according to the biochemical analysis, but a new molecular form was observed using non-reducing SDS–PAGE and native gels when the protein solution was stored for a few days at 277 K. Full dissociation into the monomeric species occurred upon addition of reducing agents, suggesting that the single cysteine residue at position 99 in DivK is involved in the formation of disulfide-mediated dimers.

Table 1
Data-collection and statistics.

Values in parentheses refer to the last shell.

Resolution (Å)	1.6
Wavelength (Å)	0.946
Oscillation (°)	2.5
No. of measurements	58069
No. of unique reflections	13114
Completeness (%)	95.1 (83.0)
$I/\sigma(I)$	6.7 (5.8)
R_{sym} (%)	6.0 (8.7)

Dynamic light-scattering experiments indicated that monomeric DivK was very sensitive to ionic strength. Reasonable particle size and apparent monodispersity were only obtained at low molarities of zwitterionic buffers. Failure to obtain crystalline material using standard screening conditions led us to investigate the physicochemical properties of the protein using small-angle X-ray scattering. The experiments were conducted at three pH values (6.0, 7.0 and 8.0) and at two protein concentrations (5 and 10 mg ml^{-1}). Monodisperse solutions of the protein were obtained in 20 m*M* MES buffer at pH 6.0 (Fig. 1) and addition of only 25 m*M* NaCl induced the formation of protein aggregates. At pH 7.0 and 8.0 in Tris buffer, the protein solution remained monodisperse up to 50 m*M* NaCl (Fig. 1). These features were

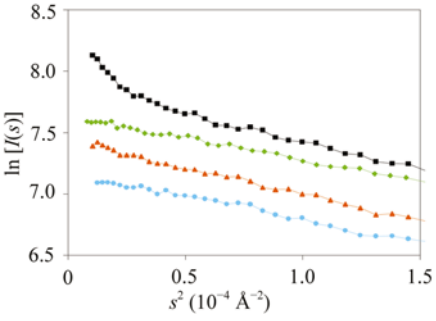

Figure 1
Guinier representation of the scattering data measured at a protein concentration of 5 mg ml^{-1}. Black squares, 20 m*M* MES, 25 m*M* NaCl pH 6; green diamonds, 20 m*M* MES pH 6; red triangles, 20 m*M* Tris, 50 m*M* NaCl pH 7; blue circles, 20 m*M* Tris, 50 m*M* NaCl pH 8.

observed at protein concentrations up to 10 mg ml^{-1}.

The average radius of gyration calculated from the Guinier plots was 16.9 $\pm$ 0.4 Å, in good agreement with the corresponding value (16.7 Å) for the homologous FixJN domain (Birck *et al.*, 1999). The extrapolated intensities $I(0)$ were brought onto an absolute scale and the calculated average molecular weight of the DivK scattering species was 14 409 Da, in good agreement with the value determined by mass spectrometry.

The conditions of monodispersity provided the basis for new crystallization trials. Thin and numerous needles were obtained using PEG-MME 2000, at pH 6.0 only, with a protein solution at 10 mg ml^{-1} in 20 m*M* MES buffer. Optimization involved the screening of temperature, protein concentration, PEG-MME molecular weight and concentration. Crystal growth occurred in narrow conditions at 285 K. It required a remarkably low protein concentration (2 mg ml^{-1}) in a limited range of 29–32%(*w*/*v*) PEG-MME 550.

The native data set was collected to 1.6 Å resolution and comprised 58 069 observations of 13 114 unique reflections (95% complete) with $R_{\rm sym}$ = 6.0% (Table 1). The symmetry of the diffracted intensities and the systematic absences indicated that the crystals belong to the orthorhombic space group $P2_12_12_1$.

Response regulators share a common doubly wound five-stranded (α/β) fold and the DivK protein displays 30.1% sequence identity with Spo0F, one of the two response regulators in the signalling cascade that controls sporulation in *B. subtilis* (Hoch & Varughese, 2001). A molecular-replacement search, using Spo0F (PDB code 1srr) as a model and the *AMoRe* suite of programs (Navaza, 1994), was performed in all possible primitive orthorhombic space groups. A distinct solution was only proposed in the $P2_12_12_1$ space group. The crystal packing indicated no major clashes in a rather tight packing, as was suggested by the crystal volume per protein mass (1.8 Å^3 Da^{-1}). Rigid-body refinement led to a crystallographic *R* factor of 53.4% at 2.9 Å but attempts to refine this model were unsuccessful. A search for suitable heavy-atom derivatives is in progress.

References

Birck, C., Mourey, L., Gouet, P., Fabry, B., Schumacher, J., Rousseau, P., Kahn, D. & Samama, J. P. (1999). *Structure Fold. Des.* **7**, 1505–1515.

Bordas, J., Koch, M. H. J., Clout, P. N., Dorrington, E., Boulin, C. & Gabriel, A. (1980). *J. Phys. E Sci. Instrum.* **13**, 939–944.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Depautex, C., Desvignes, C., Leboucher, P., Lemonnier, M., Dagneaux, D., Benoit, J. P., Vachette, P. (1987). *LURE: Rapport d'Activité pour la Période Août 1985–1986.* Documentation CEN Saclay.

Dubuisson, J. M., Descamps, T. & Vachette, P. (1997). *J. Appl. Cryst.* **30**, 49–54.

Foussard, M., Cabantous, S., Pedelacq, J. D., Guillet, V., Tranier, S., Mourey, L., Birck, C. & Samama, J. P. (2001). *Microbes Infect.* **3**, 417–424.

Guinier, A. & Fournet, G. (1955). *Small-Angle Scattering of X-rays.* New York: John Wiley & Sons Inc.

Hecht, G. B., Lane, T., Ohta, N., Sommer, J. M. & Newton, A. (1995). *EMBO J.* **14**, 3915–3924.

Hoch, J. A. & Varughese, K. I. (2001). *J. Bacteriol.* **183**, 4941–4949.

Hung, D., McAdams, H. & Shapiro, L. (2000). *Prokaryotic Development*, edited by Y. V. Brun & L. Shimkets, pp. 361–378. Washington DC: ASM Press.

Jacobs, C., Hung, D. & Shapiro, L. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 4095–4100.

Leslie, A. G. W. (1987). *Proceedings of the Daresbury Study Weekend*, edited by J. R. Helliwell, P. A. Machin & M. Z. Papiz, pp. 39–50. Warrington: Daresbury Laboratory.

Madhusudan, Zapf, J., Whiteley, M., Hoch, J. A., Xuong, N. H. & Varughese, K. I. (1996). *Structure*, **4**, 679–690.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.

Newton, A. & Ohta, N. (1990). *Annu. Rev. Microbiol.* **44**, 689–719.

Ohta, N., Grebe, T. W. & Newton, A. (2000). *Prokaryotic Development*, edited by Y. V. Brun & L. Shimkets, pp. 341–359. Washington DC: ASM Press.

Stock, A. M., Mottonen, J. M., Stock, J. B. & Schutt, C. E. (1989). *Nature (London),* **337**, 745–749.

West, A. H. & Stock, A. M. (2001). *Trends Biochem. Sci.* **26**, 369–376.

Wheeler, R. T. & Shapiro, L. (1999). *Mol. Cell*, **4**, 683–694.

Wu, J., Ohta, N. & Newton, A. (1998). *Proc. Natl Acad. Sci. USA*, **95**, 1443–1448.