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Purification, crystallization and preliminary X-ray analysis of the DNA-binding domain of AdpA, the central transcription factor in the A-factor regulatory cascade in the filamentous bacterium *Streptomyces griseus*, in complex with a duplex DNA

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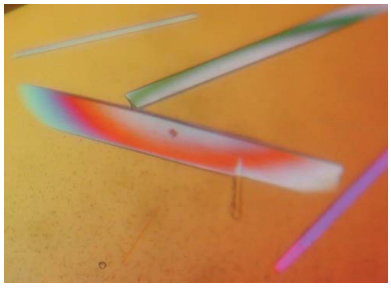
Streptomyces griseus AdpA is the central transcription factor in the A-factor regulatory cascade and activates a number of genes that are required for both secondary metabolism and morphological differentiation, leading to the onset of streptomycin biosynthesis as well as aerial mycelium formation and sporulation. The DNA-binding domain of AdpA consists of two helix–turn–helix DNA-binding motifs and shows low nucleotide-sequence specificity. To reveal the molecular basis of the low nucleotide-sequence specificity, an attempt was made to obtain cocrystals of the DNA-binding domain of AdpA and several kinds of duplex DNA. The best diffracting crystal was obtained using a 14-mer duplex DNA with two-nucleotide overhangs at the 5′-ends. The crystal diffracted X-rays to 2.8 Å resolution and belonged to space group $C222_1$, with unit-cell parameters a = 76.86, b = 100.96, c = 101.25 Å. The Matthews coefficient (V_M = 3.71 Å^3 Da^{-1}) indicated that the crystal was most likely to contain one DNA-binding domain of AdpA and one duplex DNA in the asymmetric unit, with a solvent content of 66.8%.

1. Introduction

The filamentous bacterial genus *Streptomyces* is characterized by morphological differentiation culminating in sporulation and by its ability to produce various antibiotics as secondary metabolites. In *Streptomyces griseus*, morphological differentiation and secondary metabolism are triggered by a chemical signalling molecule called A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) at an extremely low concentration (Horinouchi & Beppu, 2007; Horinouchi, 2007). AdpA is the central transcription factor in the A-factor regulatory cascade and activates a number of genes that are required for both secondary metabolism and morphological differentiation, leading to the onset of streptomycin biosynthesis as well as aerial mycelium formation and sporulation (Ohnishi *et al.*, 2005). AdpA consists of two domains, a ThiJ/PfpI/DJ-1-like dimerization domain in the N-terminal portion and a DNA-binding domain in the C-terminal portion, and belongs to the AraC/XylS family of transcriptional regulators (Yamazaki *et al.*, 2004). The C-terminal DNA-binding domain (DBD) of AdpA is characterized by two helix–turn–helix (HTH) DNA-binding motifs (Gallegos *et al.*, 1997; Tobes & Ramos, 2002). A consensus AdpA-binding sequence, 5′-TGGCSNGWWY-3′ (where S is G or C, W is A or T, Y is T or C and N is any nucleotide), was revealed by a uracil-interference assay and missing T and GA interference assays on several AdpA-binding sites followed by gel mobility-shift assays on systematically mutated binding sites (Yamazaki *et al.*, 2004). The DNA-binding specificity of AdpA is much lower than those of other well known regulators from bacteria. AdpA directly controls >500 genes; to the best of our knowledge, the AdpA regulon appears to be the largest regulon in bacteria (Higo *et al.*, 2012). The low DNA-binding specificity of AdpA enables binding to many sites on the chromosome, which facilitates the regulation of many genes. Thus, the low nucleotide-sequence specificity of the DBD of AdpA (hereafter referred to as AdpA-DBD) is a very



important feature of this global transcriptional regulator. In order to reveal the molecular mechanism of the low nucleotide-sequence specificity of AdpA-DBD, we cocrystallized AdpA-DBD with its target duplex DNAs. Here, we report a cocrystal of AdpA-DBD and a 14-mer duplex DNA that diffracted X-rays to 2.8 Å resolution.

2. Materials and methods

2.1. Overexpression and purification

The gene encoding the DBD (residues 215–340) of *S. griseus* AdpA (NCBI gene ID 6209784) was amplified by PCR from the genomic DNA of *S. griseus* and cloned into the *Nde*I/*Bam*HI site of pET-28a (Novagen) to obtain the expression plasmid for AdpA-DBD with an N-terminal 6×His tag. The deduced amino-acid sequence of the recombinant protein was MGSSHHHHHHSSGLVPRGSHM-**GQERYLDRSLPEEIGSDPLAEVVAWALEHLHEQFDVETLA-ARAYMSRRTFDRRFRSLTGSAPLQWLITQRVLQAQRLLETS-DYSVDEVAGRCGFRSPVALRGHFRRQLGSSPAAYRAAYRA-RRPQG**, where the amino-acid sequence of AdpA-DBD is shown in bold. The recombinant protein was overexpressed in *Escherichia coli* Rosetta (DE3) cells harbouring the above-mentioned expression plasmid at 298 K overnight without addition of IPTG. The cultured cells were harvested by centrifugation at 3000*g* and 277 K for 20 min. The cells were resuspended in 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 10 m*M* imidazole, 10%(*v*/*v*) glycerol on ice and disrupted by sonication. After centrifugation at 40 000*g* for 60 min, the supernatant was applied onto an Ni–NTA agarose column (Qiagen). The bound protein was washed and eluted with buffer solutions consisting of 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 10%(*v*/*v*) glycerol supplemented with 20 and 250 m*M* imidazole, respectively. The eluted protein was dialyzed against 20 m*M* MES pH 6.0, 300 m*M* NaCl, 10%(*v*/*v*) glycerol, applied onto a Resource S 6 ml column (GE Healthcare) pre-equilibrated with 20 m*M* MES pH 6.0, 300 m*M* NaCl, 10%(*v*/*v*) glycerol and eluted with a linear gradient of 300–1000 m*M* NaCl. The eluted protein was then applied onto a Superdex 75 10/30 HR column (GE Healthcare) and eluted with 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 10%(*v*/*v*) glycerol. The purified protein was dialyzed against 10 m*M* MES pH 6.0, 100 m*M* NaCl, 10%(*v*/*v*) glycerol. All column chromatography was performed at 277 K.

2.2. Preparation of the protein–DNA complex and crystallization

The oligonucleotides were purchased from Operon, Tokyo. An equimolar mixture of two complementary oligonucleotides was subjected to annealing by lowering the temperature from 368 to 277 K to obtain duplex DNAs. The AdpA-DBD–duplex DNA complexes were prepared by adding the AdpA-DBD solution to the duplex DNA solution in a 1.0:1.2 molar ratio and incubating the mixture at 277 K for 10 min. The complex solutions were then concentrated to a protein concentration of 8 mg ml^{-1} (corresponding to 500 µ*M*) using a Vivaspin 15R concentrator (molecular-weight cutoff 10 000; Sartorius). Crystallization experiments were performed at 277 K (and 283 K for optimization) using the sitting-drop vapour-diffusion method. Initial crystallization screening was performed using the commercial crystallization screening kits Crystal Screen HT, Index HT and SaltRx HT (Hampton Research) and Wizard I, II and III (Emerald BioSystems); each drop was prepared manually by mixing 0.7 µl protein–DNA complex solution with 0.7 µl reservoir solution and was equilibrated against 30 µl reservoir solution. The precipitant concentration and pH of the reservoir solution were then optimized using a two-dimensional grid screen, in which each crystallization drop was prepared by mixing 2.0 µl protein–DNA complex solution with 2.0 µl reservoir solution and was equilibrated against 200 µl reservoir solution to obtain larger crystals. Initial diffraction tests were performed using a home-source X-ray generator (Rigaku FR-E) equipped with a Rigaku R-AXIS VII detector at 100 K, using a final concentration of 20%(*v*/*v*) ethylene glycol as a cryoprotectant.

2.3. X-ray diffraction data collection and processing

Each AdpA-DBD–duplex DNA complex crystal was soaked in reservoir solution supplemented with a final concentration of 20%(*v*/*v*) ethylene glycol for several seconds for cryoprotection. Each crystal was then mounted on a cryoloop and flash-cooled to 95 K in a nitrogen stream for X-ray diffraction data collection. An X-ray diffraction data set consisting of 150 images was collected on beamline AR-NE3A at the Photon Factory, Tsukuba, Japan using X-rays with a wavelength of 1.0000 Å, an ADSC Quantum 270 CCD detector, a crystal-to-detector of 300.1 mm, a rotation angle of 1° and an exposure time of 5 s per image. Indexing, integration, scaling and merging of the diffraction data were performed with *XDS* (Kabsch, 2010).

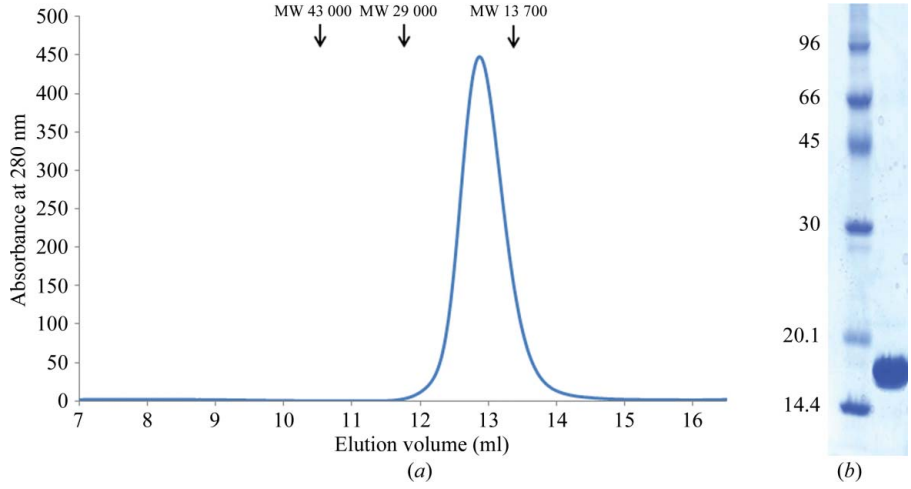


Figure 1
Purification of AdpA-DBD. (*a*) Gel-filtration chromatography on a Superdex 75 HR 10/30 column. AdpA-DBD eluted at a volume of 12.8 ml. The column was calibrated with ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and ribonuclease A (13.7 kDa) (GE Healthcare). (*b*) SDS–PAGE gel (15%) of purified AdpA-DBD. The molecular weight of AdpA-DBD is approximately 16.6 kDa. The left lane contains molecular-weight markers (labelled in kDa).

Table 1
The duplex DNAs used for cocrystallization with AdpA-DBD.

The consensus AdpA-binding sequence (10 bp) is underlined.

DNA	Nucleotide sequence	Crystallization conditions	Resolution (Å)
DNA 14-2	5′-AGGTTGGCGGGTTCAC-3′ 3′-CAACCGCCCAAGTGTC-5′	0.1 *M* sodium HEPES pH 7.1, 10%(*v*/*v*) 2-propanol, 16%(*w*/*v*) PEG 4000 (277 K)	3.3†, 2.8‡
DNA 16	5′-GGTTGGCGGGTTCACC-3′ 3′-CCAACCGCCCAAGTGG-5′	0.1 *M* sodium acetate pH 4.7, 9%(*w*/*v*) PEG 4000 (277 K)	8.0†
DNA 15-1	5′-GGTTGGCGGGTTCACC-3′ 3′-CAACCGCCCAAGTGGC-5′	0.1 *M* sodium citrate pH 5.6, 0.01 *M* $FeCl_3$, 10%(*v*/*v*) Jeffamine M-600 (277 K)	10†
DNA 18	5′-AGGTTGGCGGGTTCACCT-3′ 3′-TCCAACCGCCCAAGTGGA-5′	0.1 *M* sodium HEPES pH 7.5, 0.2 *M* NaCl, 20%(*w*/*v*) PEG 3000 (283 K)	12†
DNA 14	5′-GTTGGCGGGTTCAC-3′ 3′-CAACCGCCCAAGTG-5′	No crystal obtained	
DNA 16-2	5′-AAGGTTGGCGGGTTCACC-3′ 3′-CCAACCGCCCAAGTGGTT-5′	No crystal obtained	

† Highest resolution of the diffraction data obtained using Rigaku FR-E/R-AXIS VII. ‡ Highest resolution of the diffraction data obtained on beamline AR-NE3A at the Photon Factory.

3. Results and discussion

Recombinant AdpA-DBD was expressed in *E. coli* and was purified in three steps: immobilized Ni^{2+}-affinity, cation-exchange and gel-filtration column chromatography. The molecular weight of purified AdpA-DBD was estimated to be 16.9 kDa by gel-filtration chromatography, indicating that AdpA-DBD exists as a monomer in solution (Fig. 1*a*). SDS–PAGE analysis revealed that the purity of AdpA-DBD was greater than 95% (Fig. 1*b*). Each pair of complementary oligonucleotides was mixed and subjected to annealing to prepare the duplex DNA (Table 1). The duplex DNAs used for crystallization (Table 1) contained the consensus AdpA-binding sequence (10 bp) in the centre. Firstly, the optimal DNA length for crystallization was investigated using three kinds of duplex DNAs with blunt ends (DNAs 14, 16 and 18 in Table 1). Then, to facilitate end-to-end stacking of DNA molecules, three kinds of duplex DNAs with one- or two-nucleotide 5′-overhanging sticky ends (DNAs 15-1, 14-2 and 16-2 in Table 1) were used. The protein and DNA were mixed in a 1.0:1.2 molar ratio, concentrated to an AdpA-DBD concentration of 8.0 mg ml^{-1} (500 µ*M*) and a DNA concentration of 6.0 mg ml^{-1} (in the case of duplex DNA 14-2; 600 µ*M*) and used for crystallization. Crystals of AdpA-DBD were obtained with the duplex DNAs DNA 16, DNA 18, DNA 14-2 and DNA 15-1. The quality of each crystal was judged by its ability to diffract X-rays in two diffraction images (Table 1). Crystals of the AdpA-DBD–duplex DNA 14-2 complex, which were first obtained using condition D5 of Crystal Screen HT [0.1 *M* sodium HEPES pH 7.5, 10%(*v*/*v*) 2-propanol, 20%(*w*/*v*) PEG 4000] at 277 K in the initial screening, diffracted X-rays much better than the other crystals. By optimizing the conditions using a two-dimensional grid screen, the best crystal of the AdpA-DBD–duplex DNA 14-2 complex was obtained with a reservoir solution composed of 0.1 *M* sodium HEPES pH 7.1, 10%(*v*/*v*) 2-propanol and 16%(*w*/*v*) PEG 4000 at 277 K (Fig. 2, Table 1). The largest crystal in Fig. 2 (0.36 × 0.07 × 0.05 mm) diffracted X-rays to 2.8 Å resolution (Fig. 3, Table 1). The space group of the crystal was determined to be $C222_1$, with unit-cell parameters a = 76.86, b = 100.96, c = 101.25 Å. Data-collection statistics are summarized in Table 2. The Matthews coefficient (V_M = 3.71 $Å^3$ Da^{-1}; Matthews, 1968) indicates that the

Table 2
Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	AR-NE3A, Photon Factory
Wavelength (Å)	1.0000
Space group	$C222_1$
Unit-cell parameters	
a (Å)	76.86
b (Å)	100.96
c (Å)	101.25
Resolution (Å)	50–2.80 (2.87–2.80)
Observed reflections	53675
Unique reflections	9885
R_{merge}† (%)	7.7 (67.1)
$\langle I/\sigma(I)\rangle$	11.93 (2.19)
Completeness (%)	98.4 (93.3)
Multiplicity	5.4 (4.6)
Mosaicity (°)	0.566

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity over all equivalent reflections.

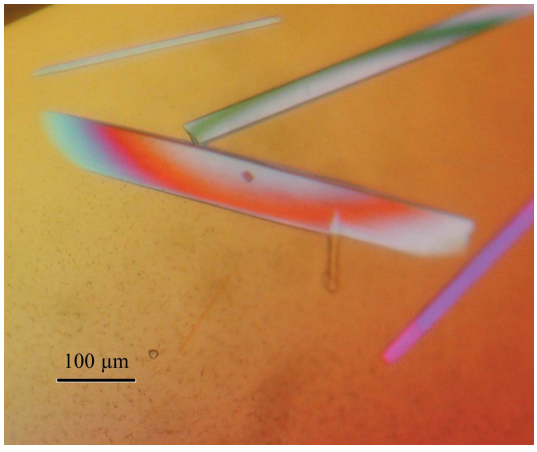


Figure 2
Crystals of the AdpA-DBD–duplex DNA 14-2 complex.

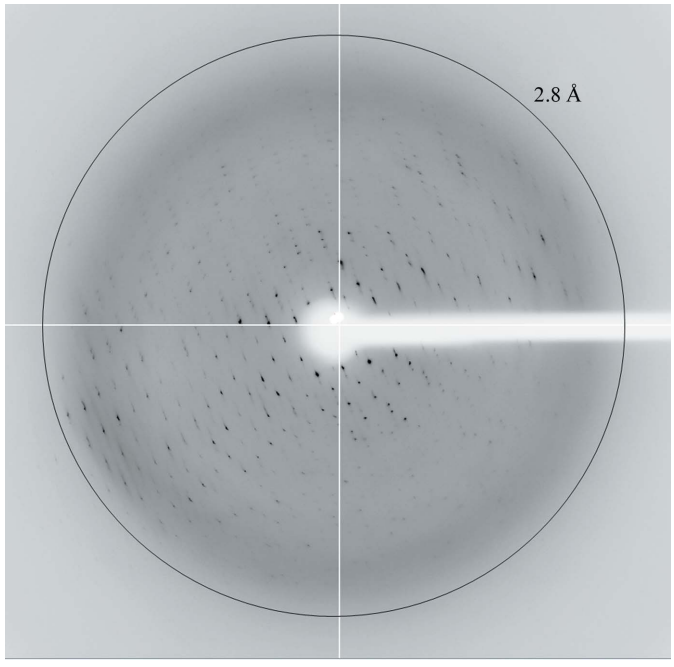


Figure 3
A diffraction image of the AdpA-DBD–duplex DNA 14-2 complex. The circle corresponds to a resolution of 2.8 Å.

crystal is most likely to contain one AdpA-DBD molecule and one duplex DNA fragment in the asymmetric unit, with a solvent content of 66.8%. Structure determination of the AdpA-DBD–duplex DNA complex is currently under way using the molecular-replacement method with the atomic coordinates of Rob (25% sequence identity; PDB entry 1d5y; Kwon *et al.*, 2000) as a search model.

The synchrotron-radiation experiments were performed on beamline AR-NE3A at the Photon Factory (Proposal No. 2008S2-001). This work was supported by the Targeted Protein Research Program (TPRP) of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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