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Crystallization and preliminary crystallographic analysis of hygromycin B phosphotransferase from *Escherichia coli*

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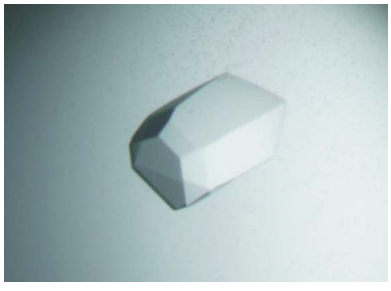

Aminoglycoside antibiotics, such as hygromycin, kanamycin, neomycin, spectinomycin and streptomycin, inhibit protein synthesis by acting on bacterial and eukaryotic ribosomes. Hygromycin B phosphotransferase (Hph; EC 2.7.1.119) converts hygromycin B to 7″-*O*-phosphohygromycin using a phosphate moiety from ATP, resulting in the loss of its cell-killing activity. The Hph protein has been crystallized for the first time using a thermostable mutant and the hanging-drop vapour-diffusion method. The crystal provided diffraction data to a resolution of 2.1 Å and belongs to space group $P3_221$, with unit-cell parameters $a = b = 71.0$, $c = 125.0$ Å. Crystals of complexes of Hph with hygromycin B and AMP-PNP or ADP have also been obtained in the same crystal form as that of the apoprotein.

1. Introduction

The aminoglycoside antibiotics target the bacterial 30S ribosomes (Pfister *et al.*, 2003) and eukaryotic ribosomes (Eustice & Wilhelm, 1984) and inhibit protein synthesis (Gonzalez *et al.*, 1978; Carter *et al.*, 2000). In particular, they exhibit strong bactericidal activity against a variety of Gram-negative bacteria as well as some Gram-positive pathogens, *e.g.* *Staphylococcus* sp. These antibiotics are therefore widely used in medicine and agriculture and increased resistance to these drugs has in turn become a problem.

Resistance to aminoglycoside antibiotics is mainly caused by enzymes that inactivate them by phosphorylation, adenylation or acetylation. Accordingly, there are three families of aminoglycoside-modifying enzymes that are responsible for antibiotic resistance: ATP-dependent *O*-phosphotransferases (APH), ATP-dependent *O*-adenyltransferases (ANT) and acetyl-CoA-dependent *N*-acetyltransferases (AAC) (Wright *et al.*, 1998; Mingeot-Leclecq *et al.*, 1999; Vaurenko & Mobasery, 2003). In order to study the structure–function relationships of these enzymes, three-dimensional structures have been determined for the following aminoglycoside-modifying enzymes belonging to the three above-mentioned families: an adenyltransferase [ANT(4′)-Ia (Sakon *et al.*, 1993)], four acetyltransferases [AAC(2′)-Ic (Vetting *et al.*, 2002), AAC(3′)-Ia (Wolf *et al.*, 1998), AAC(6′)-Ii (Wybenga-Groot *et al.*, 1999) and AAC(6′)-Iy (Vetting *et al.*, 2004)] and two phosphotransferases [APH(3′)-IIa (Nurizzo *et al.*, 2003) and APH(3′)-IIIa (Burk *et al.*, 2001)].

Among the genes for these enzymes, the phosphotransferase genes are generally used as selection markers in recombinant DNA techniques, including transgenic experiments. For example, the neomycin phosphotransferase type II gene is used in commercially available vector plasmids for neomycin or kanamycin selection. The dual substrate-recognition mechanism of this enzyme has been studied in APH(3′)-IIa and APH(3′)-IIIa (Fong & Berghuis, 2002). In contrast, the hygromycin B phosphotransferase (Hph; EC 2.7.1.119) gene, which is also used as a selection marker in plants and animals, provides resistance to only one antibiotic, hygromycin B; moreover, the three-dimensional structure of this enzyme has not been determined to date. As another example, 9-aminoglycoside phospho-

transferase type Ia [APH(9)-Ia] from *Legionella pneumophila*, which inactivates spectinomycin as a sole substrate, has been crystallized (Lemke *et al.*, 2005).

Recently, we obtained a mutant *Escherichia coli hph* gene that is intended for use as a selection marker in a thermophilic bacterium (*Thermus thermophilus*) by the directed evolution method. The mutant enzyme Hph5 contains five amino-acid substitutions, D20G, A118V, S225P, Q226L and T246A, and shows an increased thermostability of approximately 16 K *in vivo* compared with the wild-type protein (Nakamura *et al.*, 2005). In order to analyze the structure–function relationship of Hph and to further elucidate the structure-based relationships between these amino-acid substitutions and the thermostability of the enzyme, we have initiated the crystallographic study of this protein.

In this study, we report the crystallization and preliminary X-ray studies of the thermostable mutant of Hph from *E. coli*.

2. Materials and methods

2.1. Expression of *hph*5 gene in *E. coli*

The mutant *hph* gene *hph*5 was amplified using the polymerase chain reaction (PCR) from the plasmid pTEV-P31-hph5 (Nakamura *et al.*, 2005) with primers hph-f, 5′-GAATTC**CAT*ATG***AAAAAG-CCTGAACTCACCGCGACGTCT-3′, and hph-Xho-r, 5′-CC**GCTC-GAG**TTCCTTTGCCCTCGGACGAGT-3′. In the amplified DNA fragment, an *Nde*I site (bold) was introduced at the initiation codon ATG (italic) and the termination codon was replaced with an *Xho*I site (bold). The fragment was digested with *Nde*I and *Xho*I and cloned into the respective sites of pET21a(+), giving rise to pET21a-hph5. In this construct, the *hph*5 gene was fused in-frame with the 6×His tag of the plasmid at the C-terminus.

E. coli strain BL21 harbouring pET21a-hph5 and the chaperone plasmid pGro7 (Takara Shuzo Co. Ltd, Japan) was grown in 1 l Luria–Bertani medium containing ampicillin (50 µg ml^{-1}) and chloramphenicol (34 µg ml^{-1}) at 310 K to an OD_{600} of 0.6–0.8. The cultivation temperature was then shifted from 310 to 293 K, followed by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 200 µ*M* and of 0.5 mg ml^{-1} L-arabinose. The culture was maintained for a further 12 h at 293 K and the cells were harvested by centrifugation at 4850*g* for 15 min at 277 K.

Selenomethionine-substituted protein was produced using the methionine-auxotroph *E. coli* strain B834 (DE3). *E. coli* strain B834 (DE3) harbouring pET21a-hph5 was grown at 310 K in 1 l of medium containing 23.4 g SeMet core medium (Wako, Japan), 10 ml Kao and Michayluk vitamin solution (Sigma), 25 mg seleno-L-methionine and 100 ml of trace-metal mix with ampicillin (50 µg ml^{-1}) to an OD_{600} of 0.6–0.8. The cultivation temperature was then shifted from 310 to 293 K and IPTG was added to a final concentration of 200 µ*M*. The culture was maintained for a further 16 h at 293 K and the cells were harvested by centrifugation at 4850*g* for 15 min at 277 K.

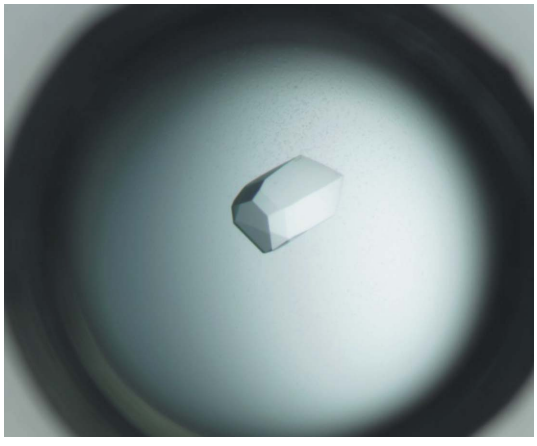

Figure 1
A micrograph of the native Hph5 crystal complexed with AMP-PNP and hygromycin B. The crystal dimensions are 0.3 × 0.2 × 0.1 mm.

2.2. Purification of Hph5

The harvested cells were suspended in buffer *A* (20 m*M* Tris–HCl, 0.5 *M* NaCl, 20 m*M* imidazole, 5 m*M* $MgCl_2$ pH 6.8) with DNase I (40 µg ml^{-1}) and phenylmethylsulfonyl fluoride (300 µg ml^{-1}). The cells were then lysed by ten cycles of sonication, each consisting of a 30 s pulse followed by 3 min cooling on ice. The cell debris was separated by centrifugation at 100 000*g* for 40 min at 277 K and the soluble fraction was applied onto an Ni^{2+}-affinity column (HiTrap chelating HP; GE Healthcare Biosciences) equilibrated with buffer *A*. The protein was eluted with a linear gradient of imidazole concentration from buffer *A* to buffer *B* (20 m*M* Tris–HCl, 0.5 *M* NaCl, 500 m*M* imidazole, 5 m*M* $MgCl_2$ pH 6.8) using a fast protein liquid-chromatography (FPLC) system (GE Healthcare Biosciences). The eluted protein was dialyzed against buffer *C* (20 m*M* Tris–HCl, 10% glycerol, 2.5 m*M* $MgCl_2$ pH 6.8) and applied onto a column of Q-Sepharose FF (GE Healthcare Biosciences) equilibrated with buffer *C*. The Hph5 protein was eluted with a linear gradient of 0–500 m*M* NaCl in buffer *C*. The purified Hph5 was concentrated using an Amicon Ultra-4 10 000 MWCO (Millipore) to 20–30 mg ml^{-1} for crystallization and stored at 193 K. Protein purification was monitored by SDS–PAGE and a single band corresponding to the Hph5 protein (341 residues and 6×His; molecular weight 38.8 kDa) was observed.

The SeMet-substituted Hph5 was purified using an Ni^{2+}-affinity column and a Q-Sepharose FF column; this process was identical to that used to purify the native Hph5 and was performed using buffers containing 20 m*M* mercaptoethanol.

2.3. Crystallization

Hph5 was crystallized by the hanging-drop vapour-diffusion method at 293 K. 1 µl Hph5 solution and 1 µl reservoir solution were mixed to form the drop. The initial crystallization conditions were examined using screening kits from Hampton Research (Crystal Screen 1, Crystal Screen 2, MembFac and PEG/Ion Screen) and Emerald Biosystems (Cryo I and II and Wizard I and II). A Hph5 crystal appeared using MembFac condition No. 1 [0.1 *M* sodium acetate pH 4.6, 0.1 *M* sodium chloride, 12%(*w*/*v*) 2-methylpentane-2,4-diol (MPD)]. The condition was further refined by changing the pH and the MPD concentration. As a result, Hph5 crystals grew to dimensions of 0.2 × 0.2 × 0.2 mm in a couple of days using the reservoir conditions 0.1 *M* sodium acetate pH 5.1–5.4, 0.1 *M* sodium chloride, 12–20%(*w*/*v*) MPD.

Crystals of Hph5 in complex with hygromycin B (Sigma) and 5-adenylyl-imidodiphosphate (AMP-PNP; Sigma) or adenosine diphosphate (ADP; Sigma) were prepared by the addition of excess amounts of hygromycin B (10 m*M*) and either AMP-PNP (5 m*M*) or ADP (5 m*M*) to the enzyme solution in buffer *C*. The complexes were formed by incubating the solution overnight at 293 K. The crystals grew in hexagonal forms and attained their full size of approximately 0.2 × 0.2 × 0.1 mm in one week (Fig. 1).

During the crystallization of SeMet Hph5, 20 m*M* mercaptoethanol was added to each reservoir. The SeMet-Hph5 crystals

appeared when using buffer conditions identical to those used for the native crystals and attained their full size of approximately 0.3 × 0.2 × 0.1 mm in one week.

2.4. Diffraction data collection

A multiple-wavelength anomalous diffraction (MAD) experiment was performed at beamline BL5A at the Photon Factory (Tsukuba, Japan), which is equipped with an ADSC Quantum-315 CCD detector. Crystals were soaked for a few minutes in reservoir solution containing 20%(*v*/*v*) MPD and were flash-frozen in a cold stream of nitrogen gas. Data collection was performed at 100 K using an oscillation angle of 1.0° and wavelengths of 0.97936 Å (peak), 0.97955 Å (edge) and 0.96432 Å (remote) (Fig. 2). Native data sets were collected from apoenzyme and complex crystals at BL5A or BL6A, Photon Factory (Tsukuba, Japan), which were equipped with an ADSC Quantum-315 CCD and an ADSC Quantum-4R CCD detector, respectively. Crystals were soaked for a few minutes in reservoir solution containing 20%(*v*/*v*) MPD and were flash-frozen in a cold stream of nitrogen gas. Data collection was performed at 100 K using an oscillation angle of 0.5° and a wavelength of 1.000 or 0.9780 Å at BL5A or BL6A, respectively (Fig. 2).

The data were processed using *MOSFLM* (Leslie, 2003) and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) and the initial phases were obtained using *SOLVE* (Terwilliger & Berendzen, 1999), which was followed by electron-density modification using *RESOLVE* (Terwilliger, 2000).

3. Results and discussion

We first attempted to crystallize wild-type Hph protein from *E. coli*; however, no crystals were obtained. Subsequently, we were successfully able to crystallize the thermostable mutant. This may be a reflection of the fact that thermostable proteins crystallize with less difficulty than wild-type proteins. Hyperthermophilic bacteria are preferred for structural genomic research owing to this property of thermostable proteins.

Recombinant *E. coli* Hph5 was produced in *E. coli* and purified to homogeneity, with a yield of approximately 12–30 mg ml^{-1} from 1 l bacterial culture. Crystals of the apo form Hph5 were reproducibly obtained in space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 71.0$, $c = 125.0$ Å. In order to examine the catalytic mechanism of Hph, we attempted to obtain crystals of Hph5 in complex with hygromycin B and AMP-PNP or ADP. The initial attempt, which used a soaking method, was unsuccessful because the crystal was broken; however, we successfully obtained crystal complexes by cocrystallization. Crystals of the same space group $P3_121$ or $P3_221$ with very similar unit-cell parameters to those of the apo form Hph5 were obtained in the presence of the ligands. This observation may

Table 1
Crystallization conditions, diffraction data and processing statistics.

Values in parentheses are for the highest resolution shell.

	SeMet apo Hph5			Native Hph5		
	Se peak	Se edge	Se remote	Apo Hph5	AMP-PNP + hygromycin B	ADP + hygromycin B
Space group	$P3_221$			$P3_221$	$P3_221$	$P3_221$
Unit-cell parameters (Å)	$a = b = 71.0$, $c = 125.0$			$a = b = 70.9$, $c = 124.6$	$a = b = 70.3$, $c = 124.1$	$a = b = 70.1$, $c = 123.8$
Molecules per ASU	1			1	1	1
Radiation source	PF BL-5A			PF BL-5A	PF BL-6A	PF BL-6A
Wavelength (Å)	0.97936	0.97955	0.96432	1.00000	0.97800	0.97800
Resolution range (Å)	62.5–2.10 (2.21–2.10)			62.4–1.90 (2.00–1.90)	41.3–1.80 (1.90–1.80)	35.0–1.50 (1.58–1.50)
Unique reflections	21978	21982	21973	29342	30433	57226
Redundancy	13.7	13.7	13.8	17.5	11.6	13.3
Completeness (%)	100 (100)	100 (100)	100 (100)	99.9 (100)	76.9 (83.3)	99.4 (100)
$I/\sigma(I)$	4.6 (2.0)	5.0 (2.0)	5.0 (2.0)	11.8 (5.5)	10.1 (3.4)	10.2 (3.7)
$R_{\rm merge}$† (%)	9.3 (37.6)	8.6 (37.7)	8.6 (38.1)	3.8 (27.7)	4.8 (30.8)	4.3 (44.4)

† $R_{\rm merge} = \sum |I_i - \langle I_i \rangle| / \sum \langle I_i \rangle$, where I_i is the observed intensity of an individual reflection and $\langle I_i \rangle$ is the mean intensity of that reflection.

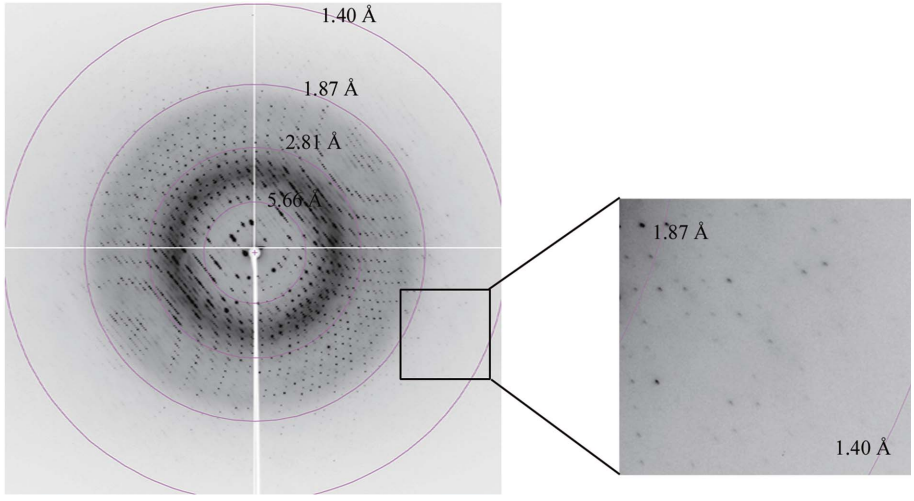

Figure 2
Diffraction pattern at beamline BL-6A, Photon Factory, Japan of a native Hph5 crystal in complex with ADP and hygromycin B obtained using reservoir solution containing MPD. The exposure time was 20 s with an oscillation angle of 0.5°. The insert shows an enlarged view of the diffraction spots in the outermost shell up to 1.4 Å.

imply that Hph5 does not undergo significant conformational change upon ligand binding, as has been suggested for APH(3′)-IIIa (Fong & Berghuis, 2004); in contrast, conformational change has frequently been observed for protein kinases (Cherry & Willams, 2004).

We have attempted to determine the structure of the apo form Hph5 using the MAD method (Hendrickson, 1991). We used a single SeMet-derivative crystal to collect a MAD data set at three wavelengths. The statistics for the diffraction data collection are given in Table 1. The data were processed using *MOSFLM* and *SCALA* and the initial phases were determined using *SOLVE*, followed by density modification with *RESOLVE*. *RESOLVE* also successfully traced the electron density to construct an initial model containing approximately 86% of the total residues. As a result, the crystals were shown to belong to space group $P3_221$. To determine the ligand-bound structures, we used the molecular-replacement method using the apo form Hph5 from this study as a search model. Further modelling and refinement are currently in progress.

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