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Crystallization and preliminary crystallographic studies of Sfp: a phosphopantetheinyl transferase of modular peptide synthetases

Mohammad R. Mofid,[a] Mohamed A. Marahiel,[a] Ralf Ficner[b] and Klaus Reuter[b]*

[a]Institut für Biochemie, Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Strasse 2, 35043 Marburg, Germany, and [b]Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Emil-Mannkopff-Strasse 2, 35037 Marburg, Germany

Correspondence e-mail: reuter@imt.uni-marburg.de

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The *Bacillus subtilis* Sfp protein is required for the non-ribosomal biosynthesis of the lipoheptapeptide antibiotic surfactin. It converts seven peptidyl carrier protein (PCP) domains of the surfactin synthetase SfrA-(A-C) to their active holo-forms by 4′-phosphopantetheinylation. The *B. subtilis sfp* gene was overexpressed in *Escherichia coli* and its gene product was purified to homogeneity and crystallized. Well diffracting single crystals were obtained from Sfp as well as from a selenomethionyl derivative, using sodium formate as a precipitant. The crystals belong to the tetragonal space group $P4_12_12/P4_32_12$, with unit-cell parameters $a = b = 65.3$, $c = 150.5$ Å. They diffract beyond 2.8 Å and contain one molecule in the asymmetric unit.

1. Introduction

Acyl carrier protein (ACP) subunits or domains as well as peptidyl carrier protein (PCP) domains are essential components of all fatty acid synthases, polyketide synthases and non-ribosomal peptide synthetases (Walsh *et al.*, 1997). Both ACPs and PCPs require a post-translational modification to become functionally active. The inactive apo-forms are converted to their active holo-forms by the transfer of the 4′-phosphopantetheinyl moiety of coenzyme A to the side-chain hydroxyl of a particular serine residue conserved in ACPs and PCPs (Marahiel *et al.*, 1997; Fig. 1). This transfer, which strongly depends on Mg^{2+} (Lambalot & Walsh, 1997), is catalyzed by a recently discovered enzyme family, the phosphopantetheinyl transferases (Lambalot *et al.*, 1996). The first such transferase to be isolated was *Escherichia coli* holo-acyl carrier protein synthase (ACPS), a 28 kDa homodimer of 125 amino-acid subunits, which converts apo-ACP of fatty acid synthase to its holo-form (Lambalot & Walsh, 1995). Subsequently, three bacterial proteins, *E. coli* EntD, *Bacillus brevis* Gsp and *B. subtilis* Sfp, consisting of 209–237 amino acids, were identified on the basis of the sequence homologies of their C-terminal halves to ACPS (Lambalot *et al.*, 1996). These proteins were shown to be essential for the biosyntheses of the Fe^{III}-chelating siderophore enterobactin and the peptide antibiotics gramicidin S and surfactin, respectively, by phosphopantetheinylating the corresponding peptide synthetases. We report here the overexpression, purification and crystallization of recombinant *B. subtilis* Sfp protein, which is essential for surfactin production. Although its physiological role is to convert the seven PCP domains of surfactin synthetase [SrfA-(A-C)] to their active holo-forms, Sfp was shown to exhibit low specificity with respect to the protein substrate. In addition to its natural substrates, it is also able to activate the ACP and PCP domains of fatty acid synthases, enterobactin synthetase (Lambalot *et al.*, 1996), bacitracin synthetase, gramicidin synthetase, tyrocidine synthetase (M. R. Mofid and M. A. Marahiel, unpublished results) and 6-methylsalicylic acid synthase (Kealey *et al.*, 1998). Sfp is the first phosphopantetheinyl transferase which has been crystallized. During all crystallization trials, coenzyme A was added in ample amounts. In the event of a successful co-crystallization of coenzyme A, the structure of Sfp is expected to provide insight into the binding mode of this substrate and the mechanism of the catalyzed reaction. It should explain the results of recently performed mutagenesis experiments (Quadri *et al.*, 1998) and provide hints about the site of interaction with the phosphopantetheinyl acceptor protein.

2. Methods, results and discussion

2.1. Cloning procedures, expression and purification

The coding region of the *sfp* gene was PCR-amplified from chromosomal DNA of *B. subtilis* strain ATCC21332 and cloned into overexpression vector pQE60 (Bujard *et al.*, 1987; QIAGEN, Germany) *via* an *Eco*RI and a *Bgl*II restriction site. The restriction sites were

introduced by the 5′ and 3′ PCR primers, respectively. The resulting plasmid pQE60-SFP encoded an Sfp protein spaced by two additional amino acids (Arg and Ser) from a C-terminal His_6 tag. It was co-transformed with the *lac* repressor encoding plasmid pREP4 (QIAGEN) into *E. coli* BL21(DE3).

BL21(DE3)(pQE60-SFP, pREP4) was cultivated in 400 ml of 2×YT medium (Sambrook *et al.*, 1989) containing 100 mg l^{-1} ampicillin and 25 mg l^{-1} kanamycin at 310 K. At $OD_{600} \simeq 0.6$, expression of the recombinant *sfp* gene was induced by addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a final concentration of 0.5 m*M*. Incubation was continued for a period of 4 h, after which cells were harvested by centrifugation. The cell pellet was resuspended in 30 ml lysis buffer (50 m*M* HEPES/NaOH, 300 m*M* NaCl, pH 8.0). Cells were disrupted at 277 K by three passes through a French pressure cell (124 MPa). The cellular debris was removed from the lysate by a 30 min centrifugation at 20000*g* and 277 K. The lysate was loaded onto a Ni–NTA–agarose (QIAGEN) column with a bed volume of 4 ml, which had been equilibrated with lysis buffer. Almost pure Sfp was eluted with a linear gradient of 0–250 m*M* imidazole (10 bed volumes) in the same buffer. The affinity chromatography step was carried out at 277 K using an FPLC system (Pharmacia, Sweden). The Sfp-containing fractions were pooled and dialyzed against 10 m*M* HEPES/NaOH (pH 8.0), 1 m*M* EDTA and 5 m*M* DTT. After dialysis, the protein solution was concentrated to 4 ml with a Centriprep 10 concentrator (Amicon, USA) and loaded onto a Superdex 75 (26/60) gel-filtration column (Pharmacia) to remove residual impurities. The column was run with 10 m*M* HEPES/NaOH (pH 8.0), 5 m*M* DTT and 120 m*M* NaCl at a flow rate of 2.5 ml min^{-1}. The gel-filtration chromatography showed that Sfp is present in a monomeric form, as calculated from calibration runs using standard proteins (data not shown). The gel-filtration step was carried out at room temperature using an Äkta Explorer system (Pharmacia). Purification yielded ∼10 mg pure Sfp from a 400 ml culture. The protein was enzymatically active (see assay below). Its identity was further confirmed by Western blot analysis using Sartoblot equipment (Sartorius) according to the protocol of the manufacturer and an Sfp-specific antiserum described by Nakano *et al.* (1992). Pure Sfp was concentrated to 10 mg ml^{-1} for crystallization experiments using a Centriprep-10 concentrator (Amicon).

2.2. Phosphopantetheinyl transferase (PPTase) activity assay

PPTase activity was monitored during Sfp purification by a qualitative assay derived from a method described by Stachelhaus *et al.* (1998): 500 n*M* truncated apo-gramicidin S synthetase 1 (GrsA), comprising the phenylalanine adenylation and the PCP domain, was incubated with 100 µ*M* coenzyme A and ∼50 n*M* Sfp in 50 m*M* Tris–HCl (pH 8.75), 10 m*M* $MgCl_2$, 2.5 m*M* DTT for 5 min at 310 K to generate the holo-form of the GrsA fragment. ^{14}C-labelled phenylalanine (453 mCi $mmol^{-1}$; Amersham, Braunschweig, Germany) and ATP were added to the reaction mixture to final concentrations of 1.25 µ*M* and 2 m*M*, respectively. The final volume of the reaction was 115 µl. After further incubation for 5 min at 310 K, the reaction was stopped by the addition of 800 µl chilled 10% trichloroacetic acid (TCA) plus 15 µl of a 25 mg ml^{-1} BSA carrier solution. The mixture was incubated on ice for 30 min. The precipitate was collected by centrifugation in a microcentrifuge at 277 K, washed three times with 10% TCA and dissolved in 180 µl of concentrated formic acid. The redissolved protein was mixed with 3.5 ml liquid-scintillation cocktail and the amount of radioactivity incorporated was quantified by liquid-scintillation counting.

2.3. Crystallization

Crystallization experiments were performed at 294 K in Linbro plates using the hanging-drop vapour-diffusion technique. Initially, a factorial screening was carried out using the 98 solutions of the commercially available Crystal Screen Kits 1 and 2 (Hampton Research, USA). A drop of 1.5 µl protein solution (10 mg ml^{-1} Sfp in 10 m*M* HEPES/NaOH pH 8.0, 5 m*M* DTT, 120 m*M* NaCl plus 1 m*M* coenzyme A) was mixed with an equal volume of reservoir solution and sealed against 1 ml reservoir solution. Coenzyme A was added to ensure homogeneity of the material to be crystallized, since about 20–30% of the purified Sfp used for crystallization contained cellular coenzyme A, as shown by mass spectrometry (see §2.5). Small crystals appeared overnight in 2.0 *M* sodium formate buffered with 100 m*M* sodium acetate at pH 4.6. In all further crystallization experiments 5 m*M* DTT and 0.02%(*w*/*v*) sodium azide were added to the reservoir solution. In the presence of sodium formate as the precipitating agent, Sfp crystallized in the pH interval between 4.5 and 6.5. The best crystals were obtained with 1.0 *M* sodium formate buffered with 100 m*M* sodium acetate at pH 5.0 in a drop prepared from 3 µl protein solution and 1.5 µl reservoir solution. The crystals were of bipyramidal shape and grew to a size of ∼0.8 × 0.5 × 0.5 mm within one week (Fig. 2). Morphologically identical crystals were obtained under the same conditions using recombinant Sfp without any C-terminal His_6 tag,

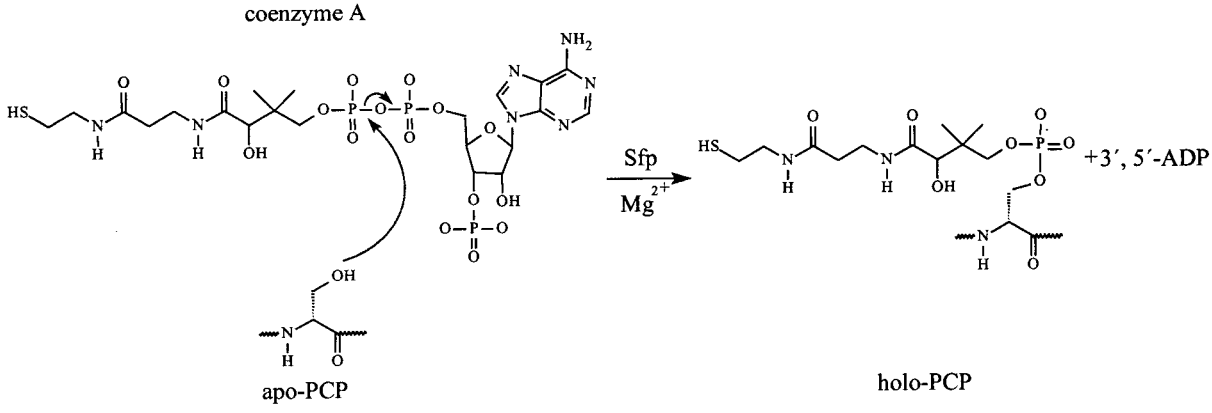

Figure 1
Reaction catalyzed by the 4′-phosphopantetheinyl transferase Sfp.

Table 1
X-ray data-collection statistics.

	Native	SeMet
Number of crystals	1	1
Resolution (Å)	30–2.8	30–2.5
Wavelength (Å)	1.54	1.54
Space group	$P4_12_12/P4_32_12$	$P4_12_12/P4_32_12$
Unit-cell parameters (Å)	$a = b = 65.34$, $c = 150.59$	$a = b = 65.19$, $c = 150.52$
Temperature of data collection	100 K	100 K
Number of observed reflections	94797	115924
Number of unique reflections	8316	11915
Completeness of all data (%)	95.3	100
R_{sym} for all data (%)	7.6	7.2
Completeness of outer shell† (%)	91.8	100
R_{sym} in outer shell† (%)	25.2	24.2

† Native, 2.90–2.80 Å; SeMet, 2.59–2.50 Å.

which had been purified by anion-exchange and hydrophobic interaction chromatography (data not shown). These crystals were not analyzed further.

2.4. X-ray diffraction experiments and crystal characterization

X-ray data were collected on an R-AXIS IV image-plate system equipped with a Rigaku RU-300 rotating-anode generator operating at 50 kV and 100 mA and focusing mirrors (MSC, USA). The crystal-to-detector distance was 130 mm and 1° oscillation images were collected with a 10 min exposure time at 100 K. Diffraction data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). To collect data under cryo-conditions, crystals were flash-frozen in a solution containing 1.0 *M* sodium formate and 100 m*M* sodium acetate (pH 5.0), with 30% glycerol as a cryo-protectant. A complete native data set was collected from a His_6-tagged Sfp crystal grown in the presence of 1 m*M* coenzyme A. The crystal diffracted to 2.8 Å and belonged to a tetragonal crystal system with unit-cell parameters $a = b = 65.34$, $c = 150.59$ Å. The space group was determined to be $P4_12_12/P4_32_12$ from systematic absences in specific reflections. Table 1 lists the data-collection statistics of the processed data set. The Matthews coefficient (V_M) (Matthews, 1968) was determined to be 2.81 Å^3 Da^{-1}, corresponding to a solvent content of 56%, assuming one Sfp and one coenzyme A molecule in the asymmetric unit.

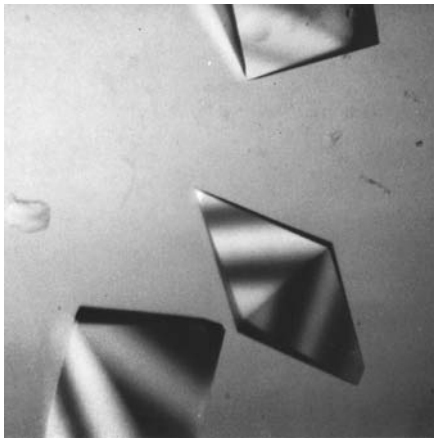

Figure 2
Crystals of the phosphopantetheinyl transferase Sfp. The crystals have approximate dimensions 0.8 × 0.5 × 0.5 mm.

2.5. Purification and crystallization of selenomethionyl Sfp

Since Sfp shares no homology with any protein of known three-dimensional structure, we intend to obtain experimental phase information by a multiple-wavelength anomalous diffraction (MAD) experiment using selenomethionine-labelled Sfp (Hendrickson *et al.*, 1990).

Therefore, *E. coli* BL21(DE3)(pQE60-SFP, pREP4) were grown in minimum medium, which was supplemented 30 min before induction with selenomethionine and ample amounts of other amino acids known to inhibit methionine biosynthesis (Van Duyne *et al.*, 1993). Purification of selenomethionyl Sfp was virtually identical to that of unlabelled Sfp and yielded ∼5 mg of pure enzymatically active selenomethionyl Sfp from a 400 ml culture. The success of selenomethionine incorporation was verified by mass spectrometry. A difference in molecular weight of 307 Da between native and selenomethionyl Sfp was measured, which corresponds to full exchange of the S atoms by Se atoms in all six of the methionines present in Sfp.

It should be noted that a second peak was observed during mass spectrometry, in addition to the main peak which corresponded well to the expected molecular mass of the native or selenomethionyl Sfp. The second peak, which amounted to some 20–30% of the total protein, indicated a molecular mass of almost exactly 767 Da above that of the main peak (data not shown). Since the molecular mass of coenzyme A is 767.5 Da, this difference strongly suggests that cellular coenzyme A was bound to a substantial portion of the Sfp protein purified for crystallization.

Selenomethionyl Sfp was crystallized under identical conditions as native Sfp. The crystals showed the same morphology, diffracted equally well and showed the same unit-cell parameters as their native counterparts. The data-collection statistics of a complete data set measured from a selenomethionyl crystal grown in the presence of 1 m*M* coenzyme A is shown in Table 1.

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