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Crystallization and preliminary X-ray crystallographic studies on acyl-(acyl carrier protein) from *Escherichia coli*

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Acyl carrier proteins carry the lipid substrate to the enzymes of the fatty acid synthase system. Crystals of *Escherichia coli* acyl carrier protein to which a butyryl group has been attached *via* a thioester link to the phosphopantetheine prosthetic arm have been obtained by the hanging-drop vapour-diffusion method. These crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 27.6, b = 41.6, c = 63.7 Å. The asymmetric unit appears to contain one subunit, corresponding to a packing density of 2.1 Å^3 Da^{-1}. Crystals of the selenomethionine-substituted (SeMet) protein were obtained using different conditions and belong to space group $P6_3$, with unit-cell parameters $a = b$ = 63.4, c = 37.0 Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ and with a monomer in the asymmetric unit (V_M = 2.5 Å^3 Da^{-1}). Crystals of a SeMet butyryl-ACP I62M variant were obtained using the conditions for the native protein. Like the native crystals, these belong to space group $P2_12_12_1$ and have unit-cell parameters a = 27.3, b = 41.9, c = 64.5 Å. A data set suitable for MAD phasing was collected from the crystals of the I62M variant to 1.8 Å resolution on the ESRF beamline ID14-4.

1. Introduction

Lipids are synthesized by the multi-functional enzyme system, fatty acid synthase (FAS), by the extension of an acetyl group attached to an acyl carrier protein (ACP). Two carbon units are added to the growing lipid chain derived from malonyl CoA in a stepwise reaction (Wakil, 1989). ACP from *E. coli* is a small monomeric protein with a molecular mass of 8847 Da (Rock & Cronan, 1979). It is composed of 77 amino acids, of which 28% are acidic with only 8% being basic (Prescott & Vagelos, 1972). ACP has a phosphopantetheine arm covalently linked to the hydroxyl group of the side chain of Ser36 and the intermediates of lipid biosynthesis are covalently linked to the sulfhydryl group at the other end of this arm (Prescott & Vagelos, 1972).

NMR studies on *holo*-ACP from *E. coli* (Holak, Kearsley *et al.*, 1988; Holak, Nilges *et al.*, 1988; Holak *et al.*, 1989; Kim & Prestegard, 1990), actinorhodin *apo*-ACP from the polyketide synthase of *Streptomyces coelicolor* (Crump *et al.*, 1997) and *apo*- and *holo*-ACP from *Bacillus subtilis* (Xu *et al.*, 2001) have revealed that the three ACPs have a common structure consisting of a four α-helical bundle, the helices of which interact to form a hydrophobic cleft or cavity capable of accommodating the prosthetic group and the thioester-bound fatty acyl chain. NMR studies have also shown that the *apo* and *holo* forms of these proteins are essentially identical and no interaction of the prosthetic group with *holo*-ACP residues other than the covalent bond to Ser36 has been detected. NMR studies on acylated ACP from *E. coli* have provided evidence of interaction between the fatty-acyl chain and the protein moiety of acyl-ACP (Mayo & Prestegard, 1985; Jones *et al.*, 1987). However, there is as yet no three-dimensional structure of ACP with a fatty-acyl substrate attached to its prosthetic group. In order to understand the manner in which acyl substrates interact with the protein moiety of ACP, we have initiated an X-ray crystallographic study of acylated forms of ACP from *E. coli*. This paper describes the crystallization and preliminary analysis of butyryl-ACP, its selenomethionine (SeMet) variant and SeMet butyryl-ACP I62M.

2. Materials and methods

2.1. Preparation and crystallization of SeMet butyryl-ACP

Initial experiments were performed with wild-type *E. coli* (K12) ACP purified *via* acid precipitation and anion-exchange chromatography (Majerus *et al.*, 1969). This was acylated in a chemical synthesis reaction using *n*-acylimidazole to add a four-carbon butyryl moiety

(Cronan & Klages, 1981). Following the successful crystallization of this acylated wild-type form of ACP (data not shown), a recombinant *E. coli* ACP was produced, purified and a butyryl ACP derivative was synthesized from the recombinant *apo*-ACP and butyryl-CoA using *holo* (ACP) synthetase (AcpS).

In order to overexpress ACP, its coding sequence was cloned into the multiple cloning site of the pET11d overexpression vector and transformed into rubidium chloride competent BL21 (DE3) *E. coli* cells. Induction of the protein was initiated in batch culture by the addition of 0.5 m*M* isopropyl thio-β-D-galactosidase (IPTG) and growth was continued for 3 h to allow ACP overproduction. The recombinant ACP was selectively released from the harvested cells by freeze–thaw extraction (Johnson & Hecht, 1994) and purified on a Porous HQ anion-exchange column using a linear lithium chloride gradient. The purified protein was reduced in the presence of 5 m*M* DTT, extensively dialysed against MQ water and freeze-dried in 5.0 mg lots.

Butyryl-ACP was synthesized from this purified material in a 2.0 ml reaction containing 350 µ*M* ACP, 700 µ*M* butyryl-CoA and 3.5 µ*M* AcpS at pH 7.3 in 50 m*M* potassium phosphate buffer. The reaction was stirred in a Reacti-Vial at room temperature for 60 min. Following synthesis, the reaction mixture was diluted to 3.5 ml and desalted through a Pharmacia PD10 desalting column into MQ water and freeze-dried. The butyryl-ACP product was confirmed by gel analyses on 18% native PAGE gel (Post-Beittenmiller *et al.*, 1991) and by accurate mass measurement on a matrix-assisted laser desorption time-of-flight mass spectrometer (MALDI-TOF).

The I62M variant of recombinant ACP was produced using the pET11D plasmid and mutagenic primers in a Quick-Change (Stratagene) mutagenesis reaction. Reactions were carried out in a total volume of 50 µl containing reaction buffer, plasmid DNA, mutagenic primers, dNTPs and *pfu* turbo polymerase in 200 µl GeneAmp (Perkin Elmer, Beaconsfield, Buckinghamshire, England) thin-walled reaction tubes. Temperature cycling was carried out in a Robocycler (Stratagene) thermal cycling PCR machine.

Selenomethionine incorporation into ACP was achieved by the method of VanDuyne *et al.* (1993). In this method, selenomethionine is incorporated into the recombinant protein by metabolic inhibition of the methionine pathway in a normal BL21 (DE3) strain. Cultures were grown in M9 plus 0.4% glucose medium without amino-acid supplements up to the point of IPTG induction. At this point, the following final concentrations of amino acids and selenomethionine were added to the cultures as solid powders: lysine (100 mg l^{-1}), phenylalanine (100 mg l^{-1}), threonine (100 mg l^{-1}), isoleucine (50 mg l^{-1}), leucine (50 mg l^{-1}), valine (50 mg l^{-1}) and selenomethionine (50 mg l^{-1}). The cultures were re-incubated at 310 K for 15 min to allow the inhibition of methionine biosynthesis to start and 0.5 m*M* IPTG was then added to induce the production of recombinant ACP and the cultures re-incubated at 310 K on an orbital shaker (150 rev min^{-1}) for 6 h. Freeze–thaw extraction, purification, confirmational gel and MALDI-TOF analyses of the SeMet ACP were as described above.

Initial crystallization trials were conducted with butyryl-ACP at 290 K by the hanging-drop vapour-diffusion method using Crystal Screen and Crystal Screen 2 kits (Hampton Research). Thin rod-like crystals were obtained from condition 45 of the Hampton Crystal Screen, which contained 18% PEG 8000, 200 m*M* zinc acetate and 100 m*M* sodium cacodylate pH 6.5. The crystallization conditions were then optimized in order to improve the crystal quality. For butyryl-ACP and the SeMet butyryl-ACP I62M variant, the best diffracting thin plate-like crystals were grown using 18–23% PEG 4000, 20 m*M* zinc acetate, 50 m*M* sodium cacodylate pH 6.0 and a protein concentration of 15 mg ml^{-1}. These crystals had maximum dimensions of 2.0 × 1.0 × 0.03 mm. The crystals of SeMet butyryl-ACP were obtained using 3 µl hanging drops containing 6 mg ml^{-1} protein mixed with the same volume of the reservoir solution containing 8–12 % PEG 20 000, 30 m*M* $ZnCl_2$ and 40 m*M* sodium cacodylate pH 6.0 and equilibrated against the reservoir solution at 290 K. These crystals had the morphology of hexagonal bipyramids, with maximum dimensions of 0.2 × 0.2 × 0.15 mm.

Table 1
Statistics of data collection.

Values in parentheses indicate values for the highest resolution shell.

	SeMet ACP I62M MAD			SeMet ACP
Space group	$P2_12_12_1$			$P6_3$
Wavelength (Å)	0.93928 (Se remote)	0.97935 (Se peak)	0.97963 (Se inflection)	0.97169
Resolution range (Å)	20.0–1.8 (1.84–1.80)	20.0–1.8 (1.84–1.80)	20.0–1.8 (1.84–1.80)	20.0–2.0 (2.05–2.00)
Completeness (%)	96 (72)	92 (53)	92 (60)	94 (90)
Observed reflections	24127	22411	22284	29701
Unique reflections	7172	6878	6877	5485
Data with $I/\sigma(I) > 3$ (%)	92 (63)	89 (55)	89 (54)	74 (33)
R_{merge}† (%)	0.049 (0.073)	0.056 (0.081)	0.051 (0.078)	0.060 (0.269)
Multiplicity	3.4	3.4	3.4	2.8

† $R_{merge} = \sum_{hkl} |I_i - I_m| / \sum_{hkl} I_m$, where I_m is the mean intensity of the reflection.

2.2. Data collection

To perform data collection at cryogenic temperatures, the crystals were soaked for 1–2 min in a cryoprotectant solution (25% PEG 4000, 20 m*M* zinc acetate, 50 m*M* sodium cacodylate pH 6.0 and 15% glycerol for the native protein and the I62M variant and 14% PEG 20 000, 30 m*M* $ZnCl_2$, 40 m*M* sodium cacodylate pH 6.0 and 25% glycerol for SeMet butyryl-ACP) and then flash-cooled in a stream of nitrogen gas at 100 K using an Oxford Cryosystems Cryostream device.

Preliminary X-ray diffraction data from the cryocooled crystals of butyryl-ACP and SeMet butyryl-ACP I62M were collected using a CCD detector on station 14.2 at the SRS Daresbury Laboratory. Diffraction data from the cryocooled crystals of SeMet butyryl-ACP were collected to 2.0 Å using a CCD detector on the ESRF beamline BM30 (wavelength 0.97 Å). A multiple-wavelength dispersion (MAD) experiment was performed on a single crystal of SeMet butyryl-ACP I62M at 100 K on the ESRF beamline ID14-4 (Grenoble, France). Complete data were collected at three wavelengths to a resolution of 1.8 Å. All the data were processed and scaled using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997) and were subsequently handled using the *CCP*4 software suite (Collaborative Computational Project, Number 4, 1994). Data-collection statistics are summarized in Table 1.

3. Results and discussion

Test data collected from a cryocooled crystal of butyryl-ACP on station 14.2 at the SRS Daresbury Laboratory showed diffraction beyond 1.4 Å. The diffraction images were

indexed using the program *DENZO* (Otwinowski & Minor, 1997), which revealed that the crystals belong to a primitive orthorhombic crystal system with unit-cell parameters a = 27.6, b = 41.6, c = 63.7 Å (crystal form *B*). Consideration of the unit-cell volume suggests that these crystals have one molecule in the asymmetric unit with a V_M of 2.1 Å^3 Da^{-1}, corresponding to a solvent content of 45% (Matthews, 1977). Examination of the diffraction patterns using the program *HKLVIEW* (Collaborative Computational Project, Number 4, 1994) showed that reflections with h, k or l odd along the h00, 0k0 or 00l axes, respectively, appear to be systematically absent, which suggests that the crystals belong to space group $P2_12_12_1$.

Analysis of the X-ray diffraction data for the crystals of SeMet butyryl-ACP using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997) is consistent with a primitive hexagonal crystal system, class 6, with unit-cell parameters $a = b$ = 148.7, c = 106.7 Å (crystal form *A*). Calculations of the Matthews coefficient suggests that these crystals have a monomer in the asymmetric unit with a V_M of 2.5 Å^3 Da^{-1}, corresponding to a solvent content of 55% (Matthews, 1977). Systematic absences were observed along the 00l axis, with only the reflections where $l = 2n$ present, suggesting that the crystals belong to space group $P6_3$. Thus, the two observed crystal forms for acyl-ACP are different to those reported previously by McRee *et al.* (1985).

Analysis of anomalous difference Patterson maps calculated using the data for the form *A* crystals of SeMet butyryl-ACP revealed a single selenium site per asymmetric unit, suggesting that one of the two methionines in the protein is disordered. In space group $P6_3$, given that the asymmetric unit has only a single anomalous scatterer, the phase ambiguity could not be resolved and we therefore introduced an additional methionine residue into the protein by site-directed mutagenesis (I62M) using a strategy for selecting propitious sites for methionine mutations (Leathy *et al.*, 1994). Following selenium substitution and butyrylation, crystals of the I62M variant were obtained in space group $P2_12_12_1$ (crystal form *B*), with unit-cell parameters a = 27.3, b = 41.9, c = 64.5 Å. Test data collected from these crystals on station 14.2 at the SRS Daresbury Laboratory showed diffraction beyond 1.3 Å.

A three-wavelength MAD experiment has been undertaken on a crystal of SeMet butyryl-ACP I62M with data collected to 1.8 Å (Table 1). Attempts to solve the structure of the butyryl-ACP using the MAD approach are currently under way.

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