Crystal structure of *Streptococcus pneumoniae* acyl carrier protein synthase: an essential enzyme in bacterial fatty acid biosynthesis

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Acyl carrier protein synthase (AcpS) catalyzes the formation of holo-ACP, which mediates the essential transfer of acyl fatty acid intermediates during the biosynthesis of fatty acids and lipids in the cell. Thus, AcpS plays an important role in bacterial fatty acid and lipid biosynthesis, making it an attractive target for therapeutic intervention. We have determined, for the first time, the crystal structure of the *Streptococcus pneumoniae* AcpS and AcpS complexed with 3′5′-ADP, a product of AcpS, at 2.0 and 1.9 Å resolution, respectively. The crystal structure reveals an α/β fold and shows that AcpS assembles as a tightly packed functional trimer, with a non-crystallographic pseudo-symmetric 3-fold axis, which contains three active sites at the interface between protomers. Only two active sites are occupied by the ligand molecules. Although there is virtually no sequence similarity between the *S.pneumoniae* AcpS and the *Bacillus subtilis* Sfp transferase, a striking structural similarity between both enzymes was observed. These data provide a starting point for structure-based drug design efforts towards the identification of AcpS inhibitors with potent antibacterial activity.

Keywords: acyl carrier protein synthase/bacterial fatty acid biosynthesis/coenzyme A/structure-based drug design/X-ray crystallography

Introduction

The emerging resistance of bacteria to antibiotics is a frightening clinical problem (Cohen, 1992; Neu, 1992; Davies, 1994; Spratt, 1994a). A number of common pathogenic bacterial species such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus*, *Shigella dysenteriae* and *Mycobacterium tuberculosis* have developed resistance to almost all of the antibiotics including β -lactams and quinolones, two of the largest and most important classes of antibiotics that have been widely prescribed for upper respiratory tract infections (Cohen, 1992; Neu, 1992; Davies, 1994; Spratt, 1994b; Tomasz and Munoz, 1995; Thomson and Sanders, 1998; Ahamed *et al.*, 1999). The β -lactam and quinolone antibiotics are known to target the biosynthesis of bacterial cell walls and DNA replication, respectively (Neu, 1992; Davies, 1994; Spratt, 1994b; Tomasz and Munoz, 1995;

Thomson and Sanders, 1998). To search for other cellular processes that can serve as antibacterial targets, in order to identify novel antibiotics that can be used to combat the current crisis of antibiotic resistance, we have concentrated on the enzymatic step of the reaction that converts apo-acyl carrier protein (apo-ACP) to holo-ACP (Elovson and Vagelos, 1968; Lam *et al.*, 1992; Lambalot and Walsh, 1995; Lambalot *et al.*, 1996; McAllister *et al.*, 2000). This reaction, catalyzed by ACP synthase (AcpS) encoded by the *acpS* gene, involves the transfer of the 4′-phosphopantetheine group of coenzyme A (CoA) to a serine residue of apo-ACP (Elovson and Vagelos, 1968; Lam *et al.*, 1992; Lambalot and Walsh, 1995; Lambalot *et al.*, 1996). The resulting holo-ACP mediates the transfer of fatty acid intermediates during the biosynthesis of fatty acids and lipids, via the covalent attachment of carboxyl groups of fatty acid intermediates to the thiol of the 4′-phosphopantetheine prosthetic group (Elovson and Vagelos, 1968; Lam *et al.*, 1992; Magnuson *et al.*, 1993; Lambalot and Walsh, 1995; Lambalot *et al.*, 1996). Therefore, this reaction is required for the biosynthesis of all bacterial fatty acids, lipid A, which is an essential component of bacterial lipopolysaccharides, and the membrane lipids, which are also derived exclusively from acyl intermediates of fatty acids (Magnuson *et al.*, 1993). The essential nature of this reaction has been well established genetically in *Escherichia coli* and *S.pneumoniae* (Lam *et al.*, 1992; Takiff *et al.*, 1992; McAllister *et al.*, 2000). Consistent with its important role in fatty acid biosynthesis, AcpS is widely present in *Mycoplasma*, and Gram-negative and Gram-positive bacteria. Thus, AcpS appears to be an attractive antibacterial target for the discovery of novel antimicrobial agents.

Escherichia coli AcpS has been well studied (Majerus *et al.*, 1965; Elovson and Vagelos, 1968; Lambalot and Walsh, 1995; Lambalot *et al.*, 1996; Gehring *et al.*, 1997; Flugel *et al.*, 2000). The *acpS* gene from *E.coli* forms an operon with the upstream gene, *pdxJ*, whose function is required for vitamin B₆ biosynthesis (Lam *et al.*, 1992; Takiff *et al.*, 1992). The *acpS* gene was originally identified as *dpj* (downstream of *pdxJ*) whose function, although unknown, was required for the growth of *E.coli* (Lam *et al.*, 1992; Takiff *et al.*, 1992). Later, the landmark biochemical study by Lambalot and Walsh (1995) led to the identification of Dpj as AcpS. *Escherichia coli* AcpS is a small, highly basic protein of ~14 kDa (Lambalot and Walsh, 1995). The *E.coli* enzyme has been purified and characterized (Lambalot and Walsh, 1995). It exhibits a broad substrate specificity and can utilize a variety of ACPs, which are required for many diverse aspects of cellular metabolism (Majerus *et al.*, 1965; Crosby *et al.*, 1995; Lambalot and Walsh, 1995; Lambalot *et al.*, 1996; Carreras *et al.*, 1997; Gehring *et al.*, 1997; Tropf *et al.*, 1998; Kutchma *et al.*, 1999; Zhou *et al.*, 1999; Flugel *et al.*,

Table I. Crystallographic data

	Inflection	Peak	Remote	Native 1	Native 2 ^a	3'5'-ADP ^a
Data collection						
energy (eV)	12 655.75	12 659.28	12 700.00	12 398.00	12 398.00	12 398.00
wavelength λ (Å)	0.979537	0.979400	0.976259	1.00000	1.00000	1.00000
no. of observations	210 097	205 948	211 823	336 325	423 425	304 946
no. of reflections	54 462	55 080	54 821	59 335	126 451	162 841
no. of unique reflections	8450	8508	8398	14 842	20 366	29 541
average multiplicity	6.4	6.5	6.5	4.0	6.2	5.5
completeness (%)						
all reflections	95/90 ^b	95/88	94/88	96/84	94/89	100/99
reflections with $I > 3\sigma$	94/85	94/86	94/84	81/57	79/53	80/44
$\langle I/\sigma \rangle$ overall	40/14	41/19	40/18	25.6/4.5	26.5/3.0	31.1/3.8
R_{merge}^c (%)	5.6/9.2	5.2/8.8	4.7/8.4	4.9/23.0	6.0/30.1	5.3/26.8
resolution (Å)	2.8	2.8	2.8	2.4	2.0	1.9
Refinement						
protein/water				2689/91	2689/146	2717/215
r.m.s. deviation from ideal geometry						
bond length (Å)/bond angles (°)				0.007/1.121	0.005/1.051	0.009/1.109
dihedral angles/improper angles (°)				22.307/0.645	22.603/0.608	21.979/0.758
Ramachandran plot statistics						
residues in most favored/allowed regions (%)				93.0/7.0	92.3/7.7	93.7/6.3
$R_{\text{work}}^d/R_{\text{free}}^{e,f}$ (%)				22.9/29.1	20.8/24.7	23.8/27.5

^aThe space group for these data sets is monoclinic $C2$, the rest of the data sets belong to orthorhombic space group $P2_12_12_1$. Both space groups contain one homotrimer per asymmetric unit.

^bData after the slash correspond to the outer shell.

^c $R_{\text{merge}} = \sum |I| - \langle I \rangle / \sum I$, where I is the intensity of an individual measurement and $\langle I \rangle$ is the mean intensity of this reflection.

^d $R_{\text{work}} = \sum |F_o - F_c| / \sum |F_o|$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

^e R_{free} is the cross-validation R -value calculated for 5% of the reflections omitted from the refinement.

^fAll reflections with $F_o > 0$ were included in the refinement.

2000). Purified AcpS also exhibits activity with a number of CoA derivatives (Gehring *et al.*, 1997). Finally, AcpS is a very low abundant protein in *E.coli* (Elovson and Vagelos, 1968; Lambalot and Walsh, 1995).

More recently, AcpS from *S.pneumoniae* has been purified and characterized (McAllister *et al.*, 2000). The *S.pneumoniae* enzyme exhibits biochemical properties similar to those of the *E.coli* AcpS. This *acpS* gene has also been shown to be essential for the growth of *S.pneumoniae* (McAllister *et al.*, 2000). As a first step towards structure-based drug design, we have determined the crystal structures of *S.pneumoniae* AcpS and the AcpS–3'5'-ADP complex at 2.0 and 1.9 Å resolution, respectively.

Results and discussion

The overall structure of apo-AcpS

To solve the structure of the *S.pneumoniae* AcpS, we purified the protein to homogeneity from an *E.coli* expression host using a three-step purification method (McAllister *et al.*, 2000). The purified AcpS was crystallized as described (Materials and methods). Crystals of AcpS were obtained after 4–5 days at room temperature. Data were collected from these crystals and the structure of AcpS was then solved by the multiple anomalous dispersion method (MAD) (Hendrickson *et al.*, 1991) using selenomethionine-substituted protein and exploiting non-crystallographic 3-fold averaging. The crystallographic data collection statistics and refinement parameters are summarized in Table I. The structure of AcpS reveals that it assembles as a tightly packed homotrimer. The overall view of the AcpS molecule is shown in

Figure 1A. The AcpS monomer has an elongated elliptical shape with approximate dimensions of $30 \times 35 \times 45$ Å (Figure 1B). The Richardson topology diagram of secondary structural elements is shown in Figure 1C. The location of these secondary structure elements within the sequence is given in Figure 2A. The AcpS structure has an α/β fold. A topology search using the SCOP program (Murzin *et al.*, 1995) did not reveal any significant similarity with AcpS. The AcpS protomer is characterized primarily by three structural motifs. The first is a classical three-stranded anti-parallel β -sheet formed by strands β_1 , β_5 and β_4 . A long α -helix packs diagonally against the β -sheet together with α -helices α_1 , α_2 , α_3 and α_4 of the anti-parallel four-helical bundle, which represents the second structural motif. The third feature consists of a long extended loop with a two-stranded anti-parallel β -sheet (β_2 and β_3). These structural motifs are organized in such a way that the long helix α_4 runs through the whole structure and is surrounded by the other structural elements.

The side chains of each helix in the four-helical bundle are arranged so that hydrophobic side chains are buried between the helices and form a hydrophobic core comprised of: Ile17, Ala20, Val21 (α_1); Phe27, Ala28, Val31, Leu32 (α_2); Met37, Phe40 (α_3); and Ile49, Leu52, Trp56 (α_4). Side chains of the residues Phe75 and Ile70 (α_5) also participate in the formation of this hydrophobic cluster. Another hydrophobic cluster is formed by the long α -helix α_4 , and the three- and two-stranded β -sheets, which contain side chains of the residues Phe62, Met66 (α_4); Phe90 (β_3); and Phe95, Ile99 (β_4).

The final refined structure of AcpS contains 115 of 124 amino acids. No electron density is observed for the first

Fig. 1. (A) Stereoview showing a ribbon diagram of the AcpS homotrimer, viewed along a non-crystallographic 3-fold axis. (B) Ribbon diagram of the C_α backbone of one *S.pneumoniae* AcpS monomer structure. (C) A topology (Richardson) diagram of AcpS. β -strands are represented as arrows, while α helices are rectangles. The secondary structure elements are defined as follows: β 1, Ile4–Glu13; α 1, Leu14–Arg23; α 2, Phe27–Val31; α 3, Ala34–Ser42; α 4, Gly45–Met66; α 5, Ile70–Leu73; β 2, Glu79–Asn82; β 3, Pro88–Gln92; β 4, Lys98–His105; β 5, Phe109–Glu117.

two N-terminal and final four C-terminal amino acids. The three protomers are related by a non-crystallographic 3-fold pseudo-symmetry axis. The three-stranded anti-parallel β -sheets of each AcpS protomer are arranged together in a barrel-like structure in the homotrimer molecule, forming a long, mostly hydrophobic tunnel that runs through the whole structure. The active sites are formed at the intermolecular interface of the homotrimer (Figure 1B) such that two protomers contribute to the formation of each active site. An active site pocket is formed by residues of helix α 4, β -strands β 4 and β 5, and loop β 2– β 3 of one protomer, and the opposite side of helix α 4, β -stranded β 5 and loop α 4– α 5 of the second protomer molecule. Since the AcpS active site is created by the interface between two monomers, which are oriented by their homotrimer architecture, we believe that the trimeric structure of AcpS is essential for activity. This is consistent with the results of dynamic light scattering

(Protein Solution Inc., Charlottesville, VA), gel filtration column chromatography and sedimentation analysis of the purified AcpS, which demonstrated AcpS as a homotrimer (McAllister *et al.*, 2000). Finally, the native structure reveals that only two out of three active sites are occupied by sulfate ions that were present in the crystallization of AcpS (Materials and methods). The sulfate ions were found to be present in the vicinity of His105, Asp10 and Lys64, which corresponds to the α -phosphate of CoA (see below).

The overall structure of the AcpS-3'5'-ADP complex

To help identify the potential active site of AcpS for a structure-based design effort, we co-crystallized the purified AcpS with CoA, a substrate of AcpS. However, to our surprise, we found that the purified AcpS was co-crystallized with 3'5'-ADP, a product of the AcpS

Fig. 2. (A) Sequence alignment of bacterial AcpS genes from different species. The most conserved regions are shown in gray. Secondary structural elements observed in the *S.pneumoniae* AcpS crystal structure are indicated above the sequence pile-up. (B) Structural alignment of two *S.pneumoniae* AcpS monomer molecules with two domains of *B.subtilis* Sfp. The N-terminal half of Sfp from Met1 to Pro103 corresponds to one protomer of the AcpS trimer (shown in blue) and has a 22% sequence identity. The C-terminal half of Sfp from Ile104 to Pro209 corresponds to a second AcpS protomer (shown in green) and has 25% sequence identity. The remaining C-terminal portion from Asp210 to Leu224 has no counterpart in the AcpS structure. The three amino acid residues involved in Mg²⁺ binding are marked by a star. The regions involved in CoA binding are marked by plus signs.

reaction. The fact that only the 3′5′-ADP moiety exhibits a well defined electron density clearly demonstrates that 3′5′-ADP, rather than CoA, was co-crystallized with AcpS. Consistent with the native structure of AcpS, the structure of the AcpS–3′5′-ADP complex shows that AcpS is a trimeric enzyme. In addition, only two out of three active sites are occupied by 3′5′-ADP in this case, rather than sulfate ions as in the case of the native structure, thus indicating that the binding of the 3′5′-ADP molecules to AcpS competes with that of the sulfate ions. These results are also in good agreement with those of gel filtration and SDS–PAGE analysis (McAllister *et al.*, 2000), which demonstrate that ACP is bound to AcpS in a 2:3 ratio. Finally, similar to that of the native structure, the unoccupied active site of the complex structure also exhibited no defined electron density for residues Ile70–Leu73 of the α4–α5 loop. Thus, the complex structure of AcpS suggests that the active form of the enzyme is trimeric.

The active site of the AcpS–3′5′-ADP complex is shown in Figure 3. The 3′5′-ADP binding site is characterized by the following structural elements. The adenine base fits in between loop β2–β3 (Gly86, Ala87 and Pro88) and loop α4–α5 (Lys64, Gly67 and Thr68) from another protomer of the AcpS trimer. An amino group of the adenine ring is in a favorable position to form a hydrogen bond with a carbonyl oxygen of Arg85 and Thr68. The ribose moiety is bound to the adenine ring with an anti-glycosidic torsion angle. The ribose is present in a 3′-endo conformation with the axial orientation of the 2′-hydroxyl and the equatorial orientation of the 3′-phosphate group. The 3′-phosphate portion of the ligand has an interaction with loop β2–β3, α4 and β4, all of which belong to the first protomer. The 3′-phosphate is surrounded by a negatively charged cluster formed by Arg39, Arg47 and Arg55. The 5′ α-phosphate is packed against β1 and α4 of the second protomer, and has hydrogen bonds with Lys64, Ser104 and His105.

Fig. 3. View of the 3'5'-ADP fragment of CoA bound to the active site of *S.pneumoniae* AcpS. The omitted electron density map corresponding to the ligand was contoured at the 1 σ level at 1.9 Å resolution.

Evolutionary fold conservation

Recently, Reuter *et al.* (1999) have reported the crystal structure of the *Bacillus subtilis* 4'-phosphopantetheinyl transferase (Sfp). Both Sfp and AcpS enzymes catalyze similar reactions (Reuter *et al.*, 1999; McAllister *et al.*, 2000). However, a sequence alignment of the *S.pneumoniae* AcpS and the *B.subtilis* Sfp did not reveal any significant similarity (Figure 2B). In addition, Sfp, a two-domain structure, is significantly larger in size than AcpS (Reuter *et al.*, 1999; McAllister *et al.*, 2000), yet they share a striking structural similarity (Figure 4). A comparison of the two (out of three) AcpS protomers with the two domain structures of Sfp indicates that the 3'5'-ADP CoA binding pocket of AcpS superimposes with the Sfp binding site. Based on the Sfp structure, the Mg^{2+} binding site of AcpS is predicted to be near the side chain of the residues Asp10, Glu12 and Glu60, along with the α - and β -phosphate groups of CoA. All three residues are identical to those present in Sfp. Further analysis of the *S.pneumoniae*, *E.coli*, *Klebsiella pneumoniae* and *Enterococcus faecium* sequences suggests that only residues Asp10 and Glu60 are strictly conserved in all four enzymes (Figure 2B).

In summary, we have determined the crystal structure of the *S.pneumoniae* AcpS, an enzyme essential for bacterial fatty acid synthesis. This enzyme catalyzes the transfer of 4'-phosphopantetheine from CoA to apo-ACP, to form holo-ACP along with 3'5'-ADP, and is an attractive target for the development of antibacterial drugs. The structure of AcpS reveals an α/β fold and demonstrates that the

trimeric structure of the enzyme appears to be essential for AcpS activity. These results represent the first structural determination of the interaction between the AcpS enzyme and its product 3'5'-ADP. These data provide a starting point for structure-based drug design efforts that should identify novel AcpS inhibitors with potent antibacterial activity.

Materials and methods

Cloning of the *S.pneumoniae* *acpS* gene, expression and purification of AcpS

The *acpS* gene was cloned from *S.pneumoniae* (a major human pathogen of the upper respiratory tract) and overexpressed in *E.coli* as described (McAllister *et al.*, 2000). The AcpS enzyme was purified from the *E.coli* expression host to homogeneity using a three-step chromatographic method as described (McAllister *et al.*, 2000).

Crystallization

Diffraction-quality crystals were grown by the vapor diffusion technique at 294K. The protein was concentrated to 8 mg/ml in a solution of 10 mM $MgCl_2$, 14 mM KCl and 20 mM Tris-HCl pH 7.1. A 4 μ l (1:1, protein/reservoir solutions) drop was equilibrated in a 500 μ l solution containing 8–15% PEG 4000, 200 mM ammonium sulfate and 100 mM citrate buffer pH 4.5. Crystals belong to orthorhombic space group $P2_12_12_1$ (unit cell parameters: $a = 49.8$ Å, $b = 59.6$ Å, $c = 114.7$ Å). Crystallization conditions similar to those described above also yield crystals that belong to monoclinic space group $C2$ (unit cell parameters: $a = 120.2$ Å, $b = 62.3$ Å, $c = 51.7$ Å, $\beta = 98.7^\circ$) for apo-AcpS (Native 2) and 3'5'-ADP complex. Both crystal forms have a homotrimeric molecule per asymmetric unit, with a V_m value (Matthews, 1968) of 2.08 Å³/Da, which corresponds to a solvent content of ~41% in both cases. CoA, in 2- to 3-fold excess of the protein, was used as a starting material for co-crystallization in the 3'5'-ADP complex.

Fig. 4. A superposition of apo-AcpS from *S. pneumoniae* (shown in blue) on the surfactin synthetase activating enzyme Sfp (4'-phosphopantetheinyl transferase) from *B. subtilis* complexed with CoA (shown in red). One protomer of AcpS is superimposed on the N-terminal domain of Sfp and a second AcpS protomer is superimposed on the C-terminal domain of Sfp. The third protomer of the AcpS trimer does not have a counterpart in Sfp structure. A sulfate ion was found in the AcpS binding site that corresponds to the position of the α -phosphate of CoA in the Sfp molecule.

Data collection, structure solution and refinement

The diffraction data were collected using a MarCCD detector on IMCA (Industrial Macromolecular Crystallography Association) beam line ID-17 at the APS (Advanced Photon Source, Argonne National Laboratories) at 100K, using 15–20% glycerol as a cryoprotectant. The diffraction data were reduced using DENZO (HKL2000) (Otwinowski and Minor, 1997) and the intensities were scaled with SCALEPACK (Collaborative Computing Project, No. 4, 1994). Most calculations were performed with the CCP4 suite of programs (Collaborative Computing Project, No. 4, 1994). MAD data at three wavelengths around the selenium K-shell edge were collected from a single crystal (Se-Met derivative) belonging to the $P2_12_12_1$ space group, at 2.8 Å resolution using the inverse beam strategy. Location of the Se sites and phasing were performed using SOLVE (Terwilliger and Berendzen, 1999), resulting in a figure of merit of 0.64 for the data in the resolution range 20–2.8 Å. Experimental phases were subsequently modified by the application of solvent flattening and 3-fold NCS averaging using the program DM (Collaborative Computing Project, No. 4, 1994). The experimental map allowed tracing of 115 amino acid residues, excluding the three-residue loop corresponding to residues Thr68–Leu73, and two N-terminal and four C-terminal residues for each monomer molecule of the homotrimer. This model was refined against data between 20 and 2.4 Å using a maximum likelihood algorithm as incorporated in the program CNX2000 (Badger *et al.*, 1999) ($R_{\text{work}} = 0.229$, $R_{\text{free}} = 0.293$) (Brünger, 1992). Subsequently, the coordinates of the trimer were used as a search model in molecular replacement (AmoRe) (Navaza, 1994) for the C2 space group crystals. This structure was refined to an R_{work} of 0.208 ($R_{\text{free}} = 0.247$) against the data in the resolution range 20–2.0 Å. The AcpS–3'5'-ADP complex structure was refined to an R_{work} of 0.236 ($R_{\text{free}} = 0.275$) for the 20–1.9 Å resolution range. The program suite QUANTA 98 (Molecular Simulation Inc., San Diego, CA) was used for visual inspection and manual corrections between rounds of refinement. An analysis of the geometry showed that all parameters were within the values expected for a model at this resolution. All residues were found in the most favorable and additionally allowed regions of a Ramachandran plot, for all three crystal structures. The first two N-terminal and the last four C-terminal residues in each AcpS protomer, and residues Ile70–Leu73 of a disordered surface loop for one of the subunits, were not defined in the electron density even after crystallographic refinement, and for this reason were not included in the final model. The overall average temperature factor of the structures was in good agreement with that calculated from the Wilson plot. Coordinates have been deposited in the PDB under entry codes IFTE, IFTF and IFTH.

The crystal structure of *B. subtilis* AcpS has been published since our manuscript was submitted to *The EMBO Journal* (Parris *et al.*, 2000).

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