Expression, purification, crystallization and preliminary X-ray analysis of the acyl carrier protein synthase (acpS) from *Mycobacterium tuberculosis*

Sidharth Chopra,[a] **Saurabh Kumar Singh,**[b] **Sushil Prasad Sati,**[b] **Anand Ranganathan**[a] **and Amit Sharma**[b]*

[a]Recombinant Gene Products Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India, and [b]Malaria Research Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India

Correspondence e-mail: asharma@icgeb.res.in

Acyl carrier protein synthase (acpS) catalyzes the formation of holo-ACP, which mediates the transfer of acyl fatty-acid intermediates during the biosynthesis of fatty acids and lipids. An expression and purification system for the *Mycobacterium tuberculosis* (Mtb) acpS has been established that yields ~15 mg l^{-1} of the enzyme in soluble form. The purified enzyme has been crystallized by the vapour-diffusion method using 2-propanol as a precipitant. The original crystal size has been improved significantly by the addition of glycerol to the mother liquor. Mtb acpS crystals belong to the space group $R3$, with unit-cell parameters $a = b = 68.53$, $c = 85.9$ Å. Native data have been collected under cryogenic conditions; phase resolution by molecular replacement and selenomethionine-aided multiwavelength anomalous dispersion techniques is ongoing.

1. Introduction

Development of resistance by bacteria to antibiotics is an alarming clinical problem. A number of common pathogenic bacterial species such as *Streptococcus pneumoniae*, *Enterococcus*, *Staphylococcus aureus*, *Shigella dysenteriae* and *Mycobacterium tuberculosis* (Mtb) have developed resistance to almost all antibiotics, including β-lactamase and quinolones, which are two of the most important antibiotics (Cohen, 1992; Neu, 1992; Davies, 1994; Spratt, 1994a,b; Ahamed *et al.*, 1999; Tomasz & Munoz, 1995). Recently, the fatty acids and polyketides of Mtb have been implicated in its virulence and pathogenesis (Cox *et al.*, 1999). We have concentrated on the enzymatic steps necessary for lipid and polyketide biosynthesis in Mtb. One key target is the enzyme that converts apo acyl carrier protein (apo-ACP) to holo-ACP (Elovson & Vagelos, 1968; McAllister *et al.*, 2000). This magnesium-dependent reaction is catalyzed by ACP synthase (acpS) and involves the transfer of the 4′-phosphopantetheine group of coenzymeA (CoA) to the active-site serine residue of apo-ACP. 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 & Vagelos, 1968; Lam *et al.*, 1992; Magnuson *et al.*, 1993; Lambalot *et al.*, 1996; Lambalot & Walsh, 1995). Thus, acpS plays an important role in bacterial fatty-acid synthesis and lipid biosynthesis and is an attractive target for development of therapeutics. Crystal structures for two bacterial acpS enzymes have recently been solved (Chirgadze *et al.*, 2000; Parris *et al.*, 2000). However, the Mtb acpS has fairly low sequence identity to the *Bacillus subtilis* acpS (~28%), and even lower identity to the *Streptococcus pneumoniae* acpS (<20%). Here, we report (i) methods for high-yield overexpression and purification of Mtb acpS, (ii) its reproducible crystallization and (iii) diffraction data analysis of Mtb acpS crystals.

2. Experimental

2.1. Cloning, overexpression and purification

The acpS gene was PCR-amplified from Mtb H37RV genomic DNA (a kind gift from Dr J. Tyagi, AIIMS, New Delhi, India) using the oligonucleotides 5′-GAATT**CATATG**GCAT-CGTCGGTGTG-3′ and 5′-AA**CTCGAG**CG-GGGCCTCCAGGATGGCGA-3′ as the forward and the reverse primers, respectively. The *Nde*I and *Xho*I restriction enzyme sites that were incorporated into the two primers are shown in bold. The amplification was carried out using standard protocols and the product was gel purified using DE-81 ion-exchange paper (Whatman). The eluted DNA fragment was cloned into the expression plasmid pET21+(c) (Novagen) using the *Nde*I and *Xho*I ends to form the acpS-pET construct, which produces a C-terminal His-tagged fusion protein.

2.2. Expression and purification of Mtb acpS

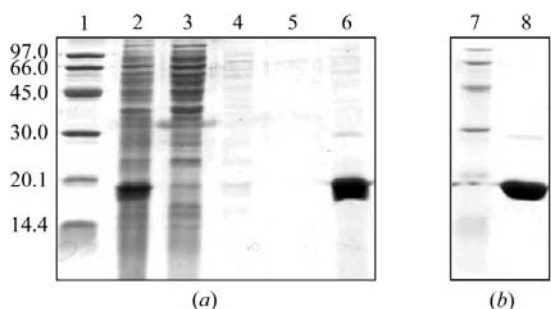AcpS-pET was introduced into *Escherichia coli* BL21 (B834 DE3) cells by heat-shock

crystallization papers

Figure 1
Expression and purification profile of Mtb acpS as judged by 15% SDS–PAGE. (*a*) Ni–NTA purification of acpS. (*b*) Gel filtration of acpS. Lanes 1 and 7, protein standards; lane 2, cell lysate; lane 3, flowthrough from Ni–NTA column; lane 4, wash; lane 5, initial eluted fraction with imidazole; lane 6, subsequent elution fraction with imidazole; lane 8, acpS after gel filtration.

transformation and the transformants were grown in Luria–Bertani (LB) broth in the presence of ampicillin (40 µg ml^{-1}). For protein production, 3 l of bacterial culture was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at an OD of ~0.6 for 4 h. The cell pellet (~12 g) was washed and resuspended in 150 ml of TES buffer saline (10 mM Tris pH 8.0, 1 mM EDTA with 0.8% saline) and was then subjected to sonication. The cell lysate obtained was clarified by centrifugation at 17 000 rev min^{-1} for 40 min in a SS-34 rotor (Sorvall). Approximately 50 ml of the clarified lysate was bound to 5 ml of pre-charged Ni–NTA (nickel–nitrilotriacetic acid) agarose (Qiagen) in TES. The resin and lysate mix was incubated at 277 K on a flip-flop for 3 h. After washing the resin with ten bed volumes of TES, acpS was eluted with 250 mM imidazole, concentrated to ~10 mg ml^{-1} and loaded onto a pre-equilibrated S-200 gel-filtration column. Eluted protein fractions were pooled and concentrated to 10 mg ml^{-1} in 10 mM Tris–HCl pH 8.0, 0.02% sodium azide and stored at 203 K. For protein estimation, the calculated extinction coefficient of acpS was used (acpS at 1 mg ml^{-1} concentration gives OD$_{280}$ = 1.31).

3. Results and discussion

3.1. Purification of acpS

The Mtb acpS can be purified to homogenity using the protocols described above (see Fig. 1). On an S-200 gel-filtration column, the protein elutes at a peak consistent with the migration of the 42 kDa oval-bumin standard, indicating that the Mtb acpS is homotrimeric in nature (the molecular weight of acpS is 15 kDa). This quaternary structure of acpS is consistent with the recent confirmation that related

acpS enzymes from various sources such as *B. subtilis* and *S. pneumoniae* are also trimeric in nature (Chirgadze *et al.*, 2000; Parris *et al.*, 2000).

3.2. Crystallization of acpS

Highly purified and concentrated acpS was used in crystallization trials based on the sparse-matrix method (Jancarik & Kim, 1991). All experiments were performed using the hanging-drop vapour-diffusion method in 24-well tissue-culture plates at room temperature. In each trial, 1 µl of protein and 1 µl of mother liquor were mixed over wells that contained 200 µl of the screening solution. Small crystals (~10 × 10 × 10 µm) of cubic morphology grew over two weeks in 0.1 M MES pH 6.5, 25% 2-propanol and 0.2 M sodium citrate. After several rounds of optimization which included changing variables such as temperature, pH, protein concentration, various alcohols, addition of substrates *etc.* it was found that the most significant improvement in crystal size was obtained only when either glycerol or glucose were used as additives. Of the two, addition of glycerol gave the largest acpS crystals (~150 × 150 × 150 µm). The crystal size and quality (*i.e.* better crystal morphology) also improved as the protein concentration was decreased from 10 to 5 mg ml^{-1}. Crystals of varying quality can be obtained with 0.1 M MES pH 6.5, 0.2 M sodium citrate, 15–25% 2-propanol and 10–20% glycerol (Fig. 2). The best crystals so far have been obtained in 0.1 M MES, 0.2 M sodium citrate, 15% 2-propanol and 10% glycerol. Addition of glycerol slows down the rate of crystal growth, resulting in crystals of improved size and morphology.

3.3. X-ray analysis of acpS crystals

A freshly grown acpS crystal was transferred into its mother liquor (supplemented with 20% glycerol), scooped into a home-made nylon loop and flash-cooled at 100 K in a nitrogen stream (Oxford Cryosystems) at the Protein Crystallography Unit of CDRI, Lucknow, India. Typically, acpS crystals were fragile and were placed in cryocooling solutions very briefly before flash-freezing (less than 30 s). Longer incubation resulted in increased mosaicity and a lower diffraction limit (~4 Å). An acpS crystal of dimensions ~100 × 100 × 100 µm was used for X-ray data collection on a Rigaku RU-300 rotating-anode X-ray

Table 1
Native diffraction data statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418
Space group	$R3$
Unit-cell parameters (Å)	$a = b = 68.53$, $c = 85.9$ Å
Resolution (Å)	15–3 (3.11–3.00)
No. of observations	5898
Unique reflections	2825
Completeness (%)	94.5 (89.9)
$I/\sigma(I)$	12.7 (3.9)
R_{sym}† (%)	6.4 (15.9)

† $R_{sym} = \sum |I_{obs} - I_{avg}| / \sum I_{obs}$, where the summation is over all reflections.

generator equipped with a Cu anode and a 345 mm image-plate detector (MAR Research, Germany). The crystal-to-detector distance was set to 300 mm, the exposure time per frame to 20 min and the oscillation range to 1° for native data collection. All data were processed, reduced and scaled with the *DENZO/SCALEPACK* programs (Otwinowski & Minor, 1997). AcpS crystals diffracted to 3.0 Å using the above setup and relevant data statistics are given in Table 1. Based on the unit-cell parameters and a molecular weight of 15 kDa, it is likely that there is one molecule of acpS per asymmetric unit in the $R3$ crystal form. This gives a solvent content of 53% and a V_M of 2.7 Å^3 Da^{-1} (Matthews, 1968).

We are attempting to use the available structures of *B. subtilis* and *S. pneumoniae* acpS as starting models for the molecular-replacement technique in order to solve the Mtb acpS structure. We have also produced selenomethionine-containing protein crystals for structure determination using MAD. The Mtb acpS structure may serve as a better template for drug-design efforts and will help in elucidating the structural basis of polyketide biosynthesis in *M. tuberculosis*.
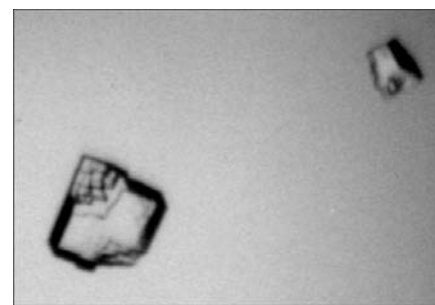

Figure 2
A typical crystal of acyl carrier protein synthase (acpS) from *M. tuberculosis*. The dimensions of the lower crystal are approximately 75 × 75 × 50 µm.

References

Ahamed, J., Gangopadhyay, J. & Sinha, A. K. (1999). *Antimicrob. Agents Chemother.* **43**, 2333–2334.

Chirgadze, N. Y., Briggs, S. L., McAllister, K. A., Fischl, A. S. & Zhao, G. (2000). *EMBO J.* **19**, 5281–5287.

Cohen, M. L. (1992). *Science*, **257**, 1050–1055.

Cox, J. S., Chen, B., McNeil, M. & Jacobs, W. R. Jr (1999). *Nature (London)*, **402**, 79–83.

Davies, J. (1994). *Science*, **264**, 375–382.

Elovson, J. & Vagelos, P. R. (1968). *J. Biol. Chem.* **243**, 3603–3611.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

Lam, H.-M., Tancula, E., Dempsey, W. B. & Winkler, M. E. (1992). *J. Bacteriol.* **174**, 1554–1567.

Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C. & Walsh, C. T. (1996). *Chem. Biol.* **3**, 923–936.

Lambalot, R. H. & Walsh, C. T. (1995). *J. Biol. Chem.* **270**, 24658–24661.

McAllister, K., Peery, R. B., Fischl, A. & Zhao, G. (2000). *J. Biol. Chem.* **275**, 30864–30872.

Magnuson, K., Jackowski, S., Rock, C. O. & Cronan, J. E. Jr (1993). *Microbiol. Rev.* **57**, 522–542.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Neu, H. C. (1992). *Science*, **257**, 1064–1073.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Parris, K. D., Lin, L., Tam, A., Mathew, R., Hixson, J., Stahl, M., Fritz, C. C., Seehra, J. & Somers, W. S. (2000). *Structure Fold. Des.* **8**, 883–895.

Spratt, B. G. (1994a). *Science*, **264**, 388–393.

Spratt, B. G. (1994b). *Bacterial Cell Wall*, edited by J. M. Ghuysen & R. Hakenbeck, pp. 517–534. Amsterdam: Elsevier.

Tomasz, A. & Munoz, R. (1995). *Microb. Drug Resist.* **1**, 103–109.