Purification and separation of *holo*- and *apo*-forms of *Saccharopolyspora erythraea* acyl-carrier protein released from recombinant *Escherichia coli* by freezing and thawing

Sandra A. MORRIS,*† W. Peter REVILL,*‡ James STAUNTON† and Peter F. LEADLAY*§

*Department of Biochemistry, and †University Chemical Laboratory, Cambridge Centre for Molecular Recognition, University of Cambridge, Cambridge CB2 1QW, U.K.

Saccharopolyspora erythraea acyl-carrier protein, highly expressed from a T7-based expression plasmid in *Escherichia coli*, can be selectively released from the cells in near-quantitative yield by a single cycle of freezing and thawing in a neutral buffer. Electrospray mass spectrometry was used to confirm that the recombinant *S. erythraea* acyl-carrier protein over-expressed in *E. coli* is present predominantly as the *holo*-form, with variable amounts of *apo*-acyl-carrier protein, *holo*-acyl-carrier protein dimer and *holo*-acyl-carrier protein glutathione adduct. The *holo*- and *apo*-acyl-carrier proteins are both readily purified on a large scale from the freeze–thaw extracts and can be separated from one another by octyl-Sepharose chromatography. The *holo*-acyl-carrier protein obtained in this way was fully active in supporting the synthesis of acyl-acyl-carrier protein by extracts of *S. erythraea*.

INTRODUCTION

Acyl-carrier protein (ACP) is a small, versatile protein that plays a central role in the biosynthesis of fatty acids in bacteria, plant chloroplasts and other organisms (see Van den Boom and Cronan, 1989, for a review). ACP is also implicated in the biosynthesis of aromatic polyketides by *Streptomyces* and related Gram-positive bacteria (reviewed by Hopwood and Sherman, 1990, as well as in the synthesis of phospholipids (Rock and Jackowski, 1982), proteins (Issartel et al., 1991), lipopolysaccharides (Brozek and Raetz, 1990) and oligosaccharides (Therisod and Kennedy, 1987). It also has an unexplained role in ATP synthesis in mitochondria (Brody and Mikolajczyk, 1988; Runswick et al., 1991; Zensen et al., 1992). The many important roles of this small protein mean that there is great interest in its structural characterization, for which it is necessary to obtain ACP from these sources in a large amount and in a pure form. The ACP that is involved in fatty-acid biosynthesis in *Escherichia coli* (molecular mass 8847 Da) is perhaps the best characterized among known ACPs (Van den Boom and Cronan, 1989). Studies carried out with this protein have shown that the form of ACP that is normally found in *E. coli* cells is *holo*-ACP, in which a 4′-phosphopantetheine prosthetic group is attached to a specific serine side-chain (Jackowski and Rock, 1983). The fact that *E. coli* ACP is produced exclusively in its functionally active (*holo*) form has facilitated its purification, and enabled enough pure protein to be obtained for structural studies using n.m.r. spectroscopy (Holak et al., 1987; Kim and Prestegard, 1990). Under certain conditions, however, the inactive or *apo*-form of ACP, which lacks the prosthetic group, may be produced in addition to the *holo*-form; for example, when the structural gene for an ACP is expressed in a heterologous system, such as in *E. coli*, the resulting recombinant protein is often observed to be expressed as a mixture both of *holo*- and of *apo*-forms (Caffrey et al., 1991; Revill and Leadlay, 1991; Shen et al., 1992). To the best of our knowledge, no effective means of separating the *holo*- and *apo*-forms of ACP on a large scale has, as yet, been developed; therefore, ACPs that are expressed as mixtures cannot be used for structural characterization. One solution to this problem has been to over-express the ACP gene in its natural host (Shen et al., 1992). However, expression in systems other than *E. coli*, such as in *Streptomyces* species, is usually more difficult and often results in lower yields of protein. A second solution to the problem is to over-express the ACP gene to high levels in *E. coli* and develop an efficient, large-scale separation of the two resulting forms of the ACP.

We have recently reported the cloning and over-expression in *E. coli* of a gene from the erythromycin-producing bacterium *Saccharopolyspora erythraea*, encoding a discrete ACP that is potentially involved in fatty-acid biosynthesis (Revill and Leadlay, 1991). In this paper we show that all forms of this ACP (as well as the *E. coli* ACP) can be selectively released in excellent yield from the recombinant cells using an osmotic-shock-release procedure. Based on this finding, a rapid chromatographic method has been developed for the preparative separation of *apo*- and *holo*-forms of the *S. erythraea* ACP.

MATERIALS AND METHODS

Materials

β-[1-^{14}C]Alanine (53 mCi/mmol) was supplied by DuPont. [2-^{14}C]Malonyl-CoA (55 mCi/mmol) was from Amersham International. All chemicals were analytical grade or alternatively were the best available.

Bacterial strains, plasmids and growth conditions

E. coli K38 (Swan et al., 1989) and plasmid pT7-7 were the gifts of Dr. S. Tabor, Harvard Medical School, Cambridge, MA, U.S.A. *E. coli* K38 contains the plasmid pGP1-2 which carries

Abbreviations used: ACP, acyl-carrier protein; DTT, dithiothreitol; e.s.m.s., electrospray mass spectrometry.

‡ Present address: John Innes Institute, John Innes Institute for Plant Science Research, Norwich NR4 7UH, U.K.

§ To whom correspondence should be addressed, at: Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 2BH, U.K.

the gene for T7 RNA polymerase, under the control of the P_L promoter (Tabor et al. 1987). Plasmid pT7-7 is a derivative of pT7-5 (Tabor and Richardson, 1985; Tabor et al., 1987) and has the strong *ϕ10* T7 promoter (Dunn and Studier, 1983) and the translation-initiation site for the T7 gene *10* protein (Dunn and Studier, 1983) inserted into pBR322, upstream of a polylinker derived from pUC12. Construction of the ACP expression plasmid pFEX-1, a pT7-7-based plasmid containing the *S. erythraea* ACP-encoding gene, has been described previously (Revill and Leadlay, 1991). *E. coli* SJ16 *panD2zad-220::Tn10* (Jackowski and Rock, 1981) was the gift of Dr. J. E. Cronan, Jr., Department of Microbiology, University of Illinois, Urbana, IL, U.S.A. Strains of *E. coli* were grown in 2 × tryptone-yeast broth [1 % (w/v) yeast extract, 1 % (w/v) tryptone, 0.5 % (w/v) NaCl] at 30 °C unless otherwise stated.

Enzyme assay and protein chemical methods

S. erythraea ACP was assayed by the method of Hale et al. (1987). Total protein was assayed either by using the dye-binding assay of Bradford (1976) with BSA as a standard, or by dialysing the protein solution against doubly deionized water adjusted to pH 7.0 with solid ammonium bicarbonate, lyophilizing and weighing the sample (final stage of purification only). Protein samples were analysed either by SDS/PAGE using the method of Laemmli (1970), using a 5 % acrylamide stacking gel and a 24 % acrylamide resolving gel, or by non-denaturing PAGE, carried out by the method of Rock and Cronan (1981). Samples for analysis by SDS/PAGE were mixed with an equal volume of standard Laemmli loading buffer (Laemmli, 1970) and boiled for 5 min before loading onto gels; samples to be analysed by non-denaturing gel electrophoresis were mixed with an equal volume of native gel-loading buffer [Tris/HCl, pH 7.5, containing 20 % (v/v) glycerol and 0.0006 % Bromophenol Blue as a tracking dye] and loaded onto the gel without previously boiling. Both the SDS/PAGE gels and the non-denaturing gels were visualized by staining with Coomassie Brilliant Blue R250. ^{14}C-labelled proteins were resolved by SDS/PAGE and stained as normal. The gels were then soaked in a solution of Amplify (Amersham International) fluorographic reagent for 15–20 min and dried on to Whatman 3MM paper under vacuum at 80 °C. Dried gels were exposed to pre-flashed Fuji RX film at −70 °C using amplifying screens. N-terminal amino-acid sequences were determined from protein bands that had been transferred to ProBlott poly(vinylidene difluoride) membranes using the method of Matsudaira (1987). Sequences were determined using an Applied Biosystems model 477A pulsed-liquid protein sequencer, fitted with an on-line model 120A analyser for the detection of phenylthiohydantoin-amino acids.

Transformation of *E. coli* strains

Cultures of *E. coli* K38 (pGP1-2) were made competent by the $CaCl_2$ method described by Sambrook et al. (1989) and stored at −80 °C. Competent *E. coli* K38 (pGP1-2) cells were transformed with pFEX-1 by the method described by Sambrook et al. (1989). Cultures of *E. coli* SJ16 (pGP1-2) were made competent by the method of Hanahan (1983). Freshly prepared competent *E. coli* SJ16 (pGP1-2) were transformed with either pFEX-1 or pT7-7 as described by Sambrook et al. (1989).

Purification of the *S. erythraea* ACP from recombinant *E. coli*

A culture of *E. coli* K38 (pGP1-2) freshly transformed with pFEX-1 was grown at 30 °C in 2 × tryptone-yeast medium with a supplement of 50 μg/ml kanamycin and 100 μg/ml ampicillin. The cells were grown to mid-exponential phase (D_{600}, 1.5–1.9), heat-shocked at 42 °C for 30 min to induce ACP production and incubated for a further hour at 30 °C. The culture (15 litres) was concentrated by centrifugation in a Heraeus continuous-action centrifuge at 4000 *g*. The cells obtained after centrifugation were stored at −80 °C for at least 24 h before ACP purification. Typically, 15 litres of culture yielded 60 g (wet weight) of cells.

All protein purification procedures were carried out at 4 °C unless otherwise indicated. Induced cells (30 g wet weight) that had been stored at −80 °C were resuspended in 600 ml of buffer A [50 mM Tris/HCl, containing 2 mM EDTA and 2 mM dithiothreitol (DTT), pH 7.5] at room temperature and gently stirred for 2 h. The cells were sedimented by centrifugation (16000 *g* for 20 min at room temperature) and both the supernatant and pellet were analysed for protein content by SDS/PAGE. The ACP was found to be exclusively in the supernatant and had a purity of greater than 85 %; the pellet was discarded. The freeze–thaw supernatant (200 mg of protein) was applied at a flow rate of 2 ml/min to a Q-Sepharose (Pharmacia) anion-exchange column (10 cm × 5 cm diameter; 200 ml bed volume). The column was washed with 200 ml of a linear gradient of 0–0.4 M KCl in buffer A, and then the bound proteins were eluted with 400 ml of a linear gradient of 0.4–0.8 M KCl in buffer A. Every third fraction (fraction size of 12 ml) was analysed by SDS/PAGE; those fractions containing the ACP (eluting at 0.6–0.65 M KCl) were pooled and dialysed for 3 h against 6 litres of buffer A (3 × 2 litres; buffer changed every hour). The dialysed sample (60 ml; 175 mg of protein) was applied at a flow rate of 1 ml/min to an octyl-Sepharose CL-4B (Pharmacia) hydrophobic-interaction column (20 cm × 3 cm diameter; 150 ml bed volume). The column was washed with 150 ml of buffer A and then 200 ml of a linear gradient of 0–30 % (v/v) propan-2-ol in buffer A. Every third fraction (fraction size of 5 ml) was analysed for ACP by non-denaturing gel electrophoresis. The *holo*-ACP eluted from the column at the end of the buffer A wash (in the absence of propan-2-ol), whereas the *apo*-ACP eluted during the gradient (eluting at 8–12 % 2-propanol). Pooled *holo*- and *apo*-ACP fractions were dialysed against doubly distilled water (made up to pH 7.0 with solid ammonium bicarbonate) and lyophilized. Typically, 100 mg of pure *holo*- and 50 mg of pure *apo*-ACP were obtained from 30 g (wet weight) of cells.

Electrospray mass spectrometry (e.s.m.s.) of ACP

Samples of *S. erythraea* ACP were analysed as previously described (Bridges et al., 1991) on a VG Bio-Q mass spectrometer consisting of an electrostatic spray ion source, operating at atmospheric pressure, attached to a quadrupole mass analyser (Loo et al., 1989). ACP samples were injected as 100 pmol/μl solutions in $MeOH/H_2O$ (1:1, v/v) containing 5 % (v/v) formic acid. Typically, 1–2 nmol of ACP was injected in each analysis. Occasionally, the ACP sample was found to have complexed with a cationic species (such as Na^+, K^+, Mg^{2+} or Ca^{2+}), causing the peaks in the mass spectrum to broaden, and rendering the molecular-mass determination very inaccurate. The ions could be removed by passing the ACP sample through a chelating resin (iminodiacetic acid) (Sigma); after this treatment, the resolution of the peaks in the mass spectrum was restored.

In vivo labelling of pantetheine-containing proteins

E. coli SJ16 (pGP1-2, pFEX-1) cells were transferred to M9 minimal medium plates (Sambrook et al., 1989), supplemented with 0.0001 % (w/v) thiamine, 40 μg/ml methionine, 100 μg/ml

ampicillin and 50 μg/ml kanamycin. Single colonies from the M9 plates were used to inoculate liquid cultures of 3 ml of the same medium, each supplemented with 5 μl (0.5 μCi) of β-[1-^{14}C]-alanine and grown at 30 °C for 48 h. The cells were pelleted and resuspended in 1 ml of medium containing a further 5 μl of β-[1-^{14}C]alanine. Cultures were heat-induced at 42 °C for 30 min and then incubated for 60 min at 30 °C. Cell lysates were prepared, analysed by SDS/PAGE and, after treatment with a fluorographic reagent, exposed to X-ray film as described above.

Freeze–thaw analysis of *in vivo*-labelled pantetheine-containing proteins

Cultures of 3 ml both of *E. coli* SJ16 (pGP1-2, pFEX-1) and of *E. coli* SJ16 (pGP1-2, pT7-7) were grown as described above. The cultures of 3 ml were used to inoculate 50 ml of liquid M9 medium supplemented with 0.0001 % (w/v) thiamine, 40 μg/ml methionine, 100 μg/ml ampicillin, 50 μg/ml kanamycin and 75 μl (12.5 μCi) of β-[1-^{14}C]alanine. After growing the cultures at 30 °C for 48 h, the cells were pelleted (4000 ***g*** for 10 min at 4 °C) and resuspended in 10 ml of fresh medium containing a further 75 μl of β-[1-^{14}C]alanine. The cultures were heat-induced at 42 °C for 30 min and then incubated for 60 min at 30 °C. The cells were pelleted (4000 ***g*** for 20 min at 4 °C; approx. 100 mg of cells from each culture), frozen at −80 °C for 24 h and then thawed into 2 ml of buffer A with gentle stirring. The freeze–thawed cells were sedimented by centrifugation (16000 ***g*** for 20 min at room temperature) and both the pellet and supernatant were analysed by SDS/PAGE and, after treatment with a fluorographic reagent, exposed to X-ray film as described above.

RESULTS AND DISCUSSION

In this study, the *S. erythraea* ACP over-expressed in *E. coli* K38 (pGP1-2) was first checked for correct post-translational processing by introducing the plasmids pGP1-2 and pFEX-1 into *E. coli* strain SJ16 which is a *panD* mutant defective in the biosynthesis of β-alanine, a precursor of 4′-phosphopantetheine (Cronan, 1980). The cells were starved of β-alanine by growth on M9 minimal medium plates. The starved cells were then used to inoculate liquid cultures of M9 medium which included β-[1-^{14}C]alanine. The cells were heat-induced following the protocol described in this paper for *E. coli* K38 (pGP1-2, pFEX-1). Cell extracts were analysed by SDS/PAGE and autoradiography.

Two proteins were intensely labelled in the induced *E. coli* and only one protein in the uninduced cells (see Figure 1). The band which appears in both, at approx. 20 kDa, corresponds with labelled *E. coli* ACP (Guerra et al., 1988); this protein, although it has a molecular mass of 8847 Da, runs anomalously high in SDS/PAGE analyses because it binds less SDS than do typical globular proteins (Rock and Cronan, 1979). The heavily labelled band at approx. 6 kDa in Figure 1(b), lane 2, corresponds with *S. erythraea* ACP, confirming that this protein is, at least to some extent, correctly post-translationally modified by *E. coli* (Revill and Leadlay, 1991). The identity of the *S. erythraea* ACP was confirmed by N-terminal sequence analysis.

The purification procedure described previously for *S. erythraea* ACP (Revill and Leadlay, 1991) consisted of mechanical cell disruption to release cellular proteins, a precipitation step and two chromatographic separations. The final purification step, Mono-Q HR (Pharmacia) anion-exchange chromatography, resulted in a significant loss of ACP and did not permit the separation of *apo*- and *holo*-ACP. In the present study, we describe both a rapid procedure for selectively releasing ACP from frozen, heat-induced *E. coli* cells and a chromatographic method for separating the *apo*- and *holo*-forms of the *S. erythraea* ACP. The first step of the purification, the selective release of ACP by a single cycle of freezing and thawing into a reducing buffer, was developed based on the observation by a number of researchers that certain cytoplasmic *E. coli* proteins, such as thioredoxin and ribosomal elongation factor Tu, can be selectively and gently released from the cells in a near-quantitative yield by a process of osmotic shock (Nossal and Heppel, 1966;

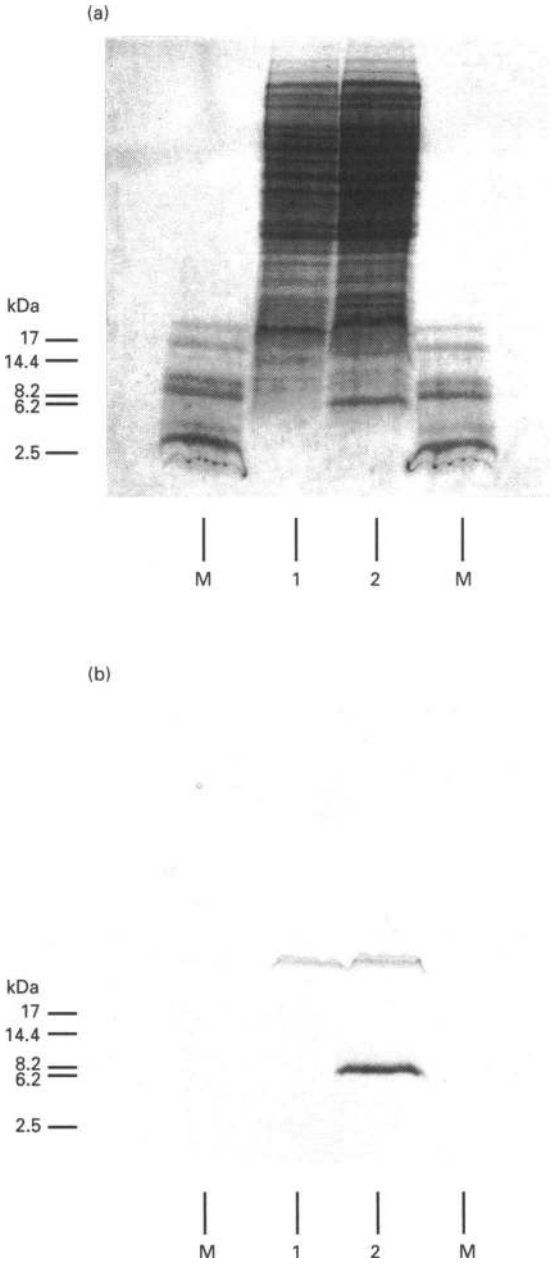

Figure 1 (a) SDS/24% PAGE analysis of whole-cell lysates of induced and uninduced *E. coli* SJ16 (pGP1-2, pFEX-1) cells grown in the presence of β-[^{14}C]alanine and (b) autoradiogram corresponding to the SDS/PAGE analysis shown in (a)

Visualization in (**a**) is by Coomassie Blue staining. Lanes M, low-molecular-mass protein markers (kDa); lane 1, uninduced cells; lane 2, induced cells.

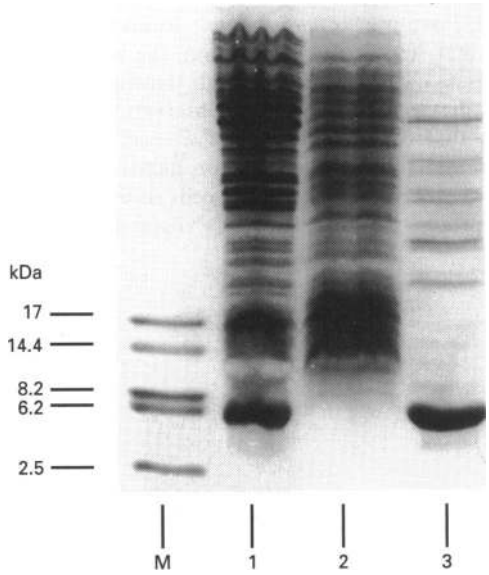

Figure 2 SDS/24% PAGE analysis of *E. coli* K38 (pGP1-2, pFEX-1) at various stages of purification of ACP

Visualization is by Coomassie Blue staining. Lane M, low-molecular-mass protein markers (kDa); lane 1, whole-cell lysate of induced cells; lane 2, pellet remaining after freeze–thaw procedure; lane 3, supernatant remaining after freeze–thaw procedure.

Figure 3 Non-denaturing PAGE analysis of ACP mixture remaining in the freeze–thaw supernatant under non-reducing conditions

Visualization is by Coomassie Blue staining. A, *apo*-ACP; B, *holo*-ACP; C, *holo*-ACP dimer; D, *holo*-ACP glutathione adduct.

Jacobson et al., 1976; Lunn and Pigiet, 1982). Normally, the osmotic-shock release of these proteins requires the presence of an osmotic stabilizer such as sucrose (up to 40%, w/v); however, for the *E. coli* protein thioredoxin, release could be achieved by a more simple freeze–thaw procedure in which the cell pellets were resuspended in a 50 mM Tris/3 mM EDTA buffer (pH 7.4), quickly frozen in a dry ice/ethanol bath and slowly thawed again on ice (Lunn and Pigiet, 1982). More recently, it has been found that certain recombinant proteins that have been over-expressed in the cytoplasm of *E. coli* can be released by an osmotic-shock procedure (Joseph-Liauzun et al., 1990; LaVallie et al., 1993). Human recombinant interleukin-1β can be isolated from *E. coli* by simple osmotic shock into a 15% (w/v) sucrose solution (Joseph-Liauzun et al., 1990) and mammalian proteins expressed as fusions with *E. coli* thioredoxin can be released either by osmotic shock into a sucrose solution or by the simple freeze–thaw procedure that releases thioredoxin itself (LaVallie et al., 1993). The cellular location of the *E. coli* cytoplasmic proteins that are selectively released by osmotic shock is thought to be the main determinant in their ability to be released by this method; both thioredoxin and elongation factor Tu are associated with the inner surface of the cytoplasmic membrane (Jacobson and Rosenbusch, 1976; Lunn and Pigiet, 1982). Since evidence has been reported that *E. coli* ACP is associated with the cytoplasmic membrane (Van der Bosch et al., 1970; Bayan and Therisod, 1989), it was thought that it too might be selectively released from *E. coli* by osmotic shock.

Attempts to purify the *S. erythraea* ACP by osmotic-shock release into various concentrations of sucrose solutions (15–40%, w/v) were unsuccessful. Only small amounts of impure ACP were released. However, it was found that if the induced cells were immediately frozen at −80 °C after harvesting, kept at this temperature for 24 h or more and then thawed directly into buffer A at room temperature with gentle stirring, the ACP was released from the cells at greater than 85% purity. Centrifugation of the cell suspension and then SDS/PAGE analysis of the proteins both in the supernatant and in the pellet showed that the release of ACP by this method was essentially quantitative if approx. 20 ml of buffer per g of cells were used (see Figure 2). To determine if this procedure might be applied to ACPs other than the *S. erythraea* ACP, *E. coli* SJ16 (pGP1-2, pFEX-1) and *E. coli* SJ16 (pGP1-2, pT7-7) cultures that had been labelled with β-[1-^{14}C]alanine were subjected to the freeze–thaw process. The results of these experiments showed that both the *S. erythraea* and the *E. coli* ACPs could be released into the supernatant by the freeze–thaw procedure. The proportion of *S. erythraea* ACP released into the supernatant was approximately equal to the proportion of *E. coli* ACP released. These results do not necessarily support the proposal (Van der Bosch et al., 1970; Bayan and Therisod, 1989) that ACP is associated with the cytoplasmic membrane, but they do indicate that the freeze–thaw method of releasing ACPs from *E. coli* cells may be of general applicability.

In the first instance, the induced *E. coli* K38 (pGP1-2, pFEX-1) cells were thawed into buffer A that contained no DTT. When the ACP that had been released into the non-reducing freeze–thaw buffer was analysed by native PAGE, four species were observed (see Figure 3) (proteins other than ACP-derived species were always observed to remain in the stacker gel and, therefore, crude ACP preparations could be analysed by non-denaturing PAGE). The mixture was analysed by e.s.m.s. and only three species of differing molecular masses were detected (see Figure 4). The measured molecular masses corresponded with *apo*-ACP (measured, 10422.2 Da; calculated, 10422.7 Da), *holo*-ACP (measured, 10763.6 Da; calculated, 10762.0 Da) and a glutathione adduct of *holo*-ACP (measured, 11068.2 Da; calculated, 11067.3 Da). Glutathione adducts of ACP have been previously reported (Butt and Ohlrogge, 1991). The identity of the fourth species that was observed by non-denaturing PAGE was not directly revealed by e.s.m.s.; however, the position of band C (Figure 3) with respect to those of the other ACP species on the non-denaturing gel suggested that the species to which it corresponded was a *holo*-ACP dimer (Rock and Cronan, 1981), a species that is indistinguishable from *holo*-ACP by e.s.m.s. To support the proposal that two of the four species that were observed by non-denaturing PAGE were *holo*-ACP disulphide adducts, the ACP mixture was incubated at 30 °C for 2 h in

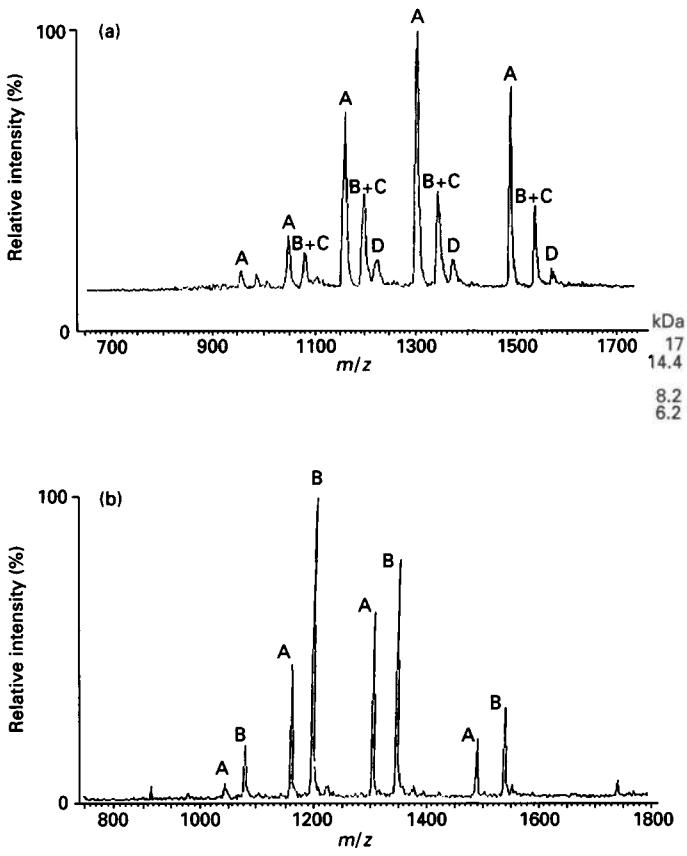

Figure 4 Electrospray mass spectra of ACP mixtures

(**a**) Mixture of ACPs remaining in the freeze–thaw supernatant under non-reducing conditions (peak A, measured, 10422.2 Da; calculated for *apo*-ACP, 10422.7 Da; peaks B + C, measured, 10763.6 Da; calculated for *holo*-ACP, 10762.0 Da; peak D, measured, 11068.2 Da; calculated for *holo*-ACP glutathione adduct, 11067.3 Da); (**b**) the same mixture as in (**a**) after treatment with DTT (peak A, measured, 10423.3 Da; calculated for *apo*-ACP, 10422.7 Da; peak B, measured, 10762.4 Da; calculated for *holo*-ACP, 10762.0 Da).

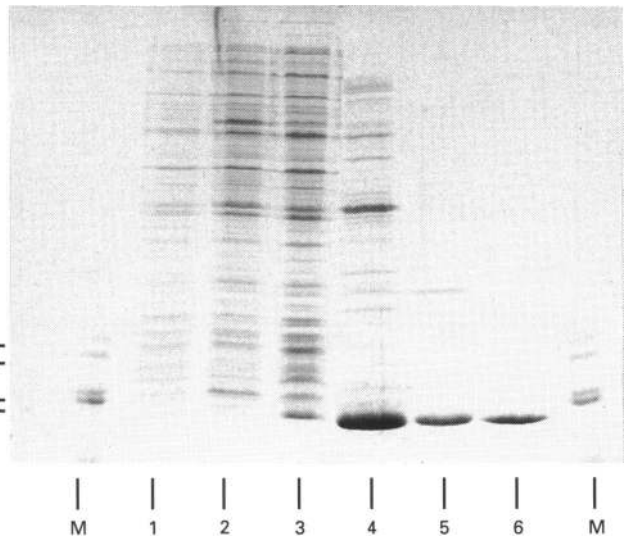

Figure 5 SDS/24% PAGE analysis of *E. coli* K38 (pGP1-2, pFEX-1) at various stages of purification of ACP

Visualization is by Coomassie Blue staining. Lanes M, low-molecular-mass protein markers (kDa); lane 1, whole-cell lysate of uninduced *E. coli* K38 (pGP1-2) without pFEX-1; lane 2, whole-cell lysate of uninduced *E. coli* K38 (pGP1-2, pFEX-1); lane 3, whole-cell lysate of induced *E. coli* K38 (pGP1-2, pFEX-1); lane 4, supernatant remaining after freeze–thaw procedure; lane 5, pooled fractions after anion-exchange chromatography on Q-Sepharose; lane 6, pooled fractions after hydrophobic-interaction chromatography on octyl-Sepharose CL-4B.

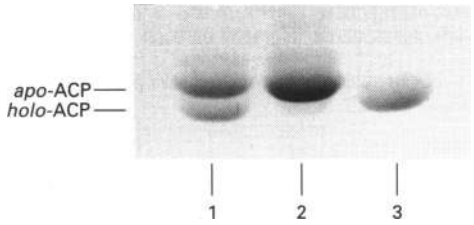

Figure 6 Non-denaturing PAGE analysis of ACP following the last two steps of purification

Visualization is by Coomassie Blue staining. Lane 1, pooled fractions after anion-exchange chromatography on Q-Sepharose; lane 2, pooled late-eluting ACP fractions from hydrophobic-interaction chromatography on octyl-Sepharose CL-4B; lane 3, pooled early-eluting ACP fractions from hydrophobic-interaction chromatography on octyl-Sepharose CL-4B.

buffer A (which contains 2 mM DTT). The resulting mixture of ACPs was again analysed by non-denaturing PAGE and e.s.m.s. Non-denaturing PAGE analysis of the mixture revealed only two bands, corresponding with those previously labelled as A and B in Figure 3. The electrospray mass spectrum of the reduced ACP mixture also showed only two species, one with a molecular mass corresponding with *apo*-ACP (measured, 10423.3 Da; calculated, 10422.7 Da) and the other with *holo*-ACP (measured, 10762.4 Da; calculated, 10762.0 Da; see Figure 4). This identified bands A and B on the native gel as *apo*- and *holo*-ACP respectively. Bands C and D were assigned as the *holo*-ACP dimer and the glutathione adduct respectively.

To simplify purification, the freeze–thaw process and all subsequent purification steps were carried out in the presence of DTT. Thus, after being released from the cells, the ACP had a greater than 85% purity and consisted only of *apo*- and *holo*-forms. The freeze–thaw supernatant was loaded directly on to a Q-Sepharose anion-exchange column and eluted as described above. The protein fraction eluting at 0.6–0.65 M KCl contained ACP that was contaminated by one other protein (see Figure 5). All the fractions containing ACP were pooled, since the analysis of these fractions showed that no separation of the *holo*- and *apo*-ACP had occurred (see Figure 6). The final purification of the ACP was achieved by chromatography on octyl-Sepharose CL-4B. Rock and Garwin had shown that *E. coli holo*-ACP could be separated from acylated ACP species using octyl-Sepharose chromatography (Rock and Garwin, 1979). They observed that the *holo*-ACP would not bind to octyl-Sepharose CL-4B under any conditions, whereas the acylated ACP derivatives bound tenaciously to it and could only be eluted using high concentrations of propan-2-ol (25%, w/v) in the elution buffer. Since it was possible that the 4′-phosphopantetheine prosthetic group might render the *holo*-ACP slightly more hydrophilic than the *apo*-ACP, we attempted to separate the two species by chromatography on octyl-Sepharose CL-4B, which is very strongly hydrophobic. The fractions pooled after the Q-Sepharose purification step were dialysed against buffer A, and applied directly to the octyl-Sepharose column. The contaminant protein remaining after the Q-Sepharose chromatography was found to elute during

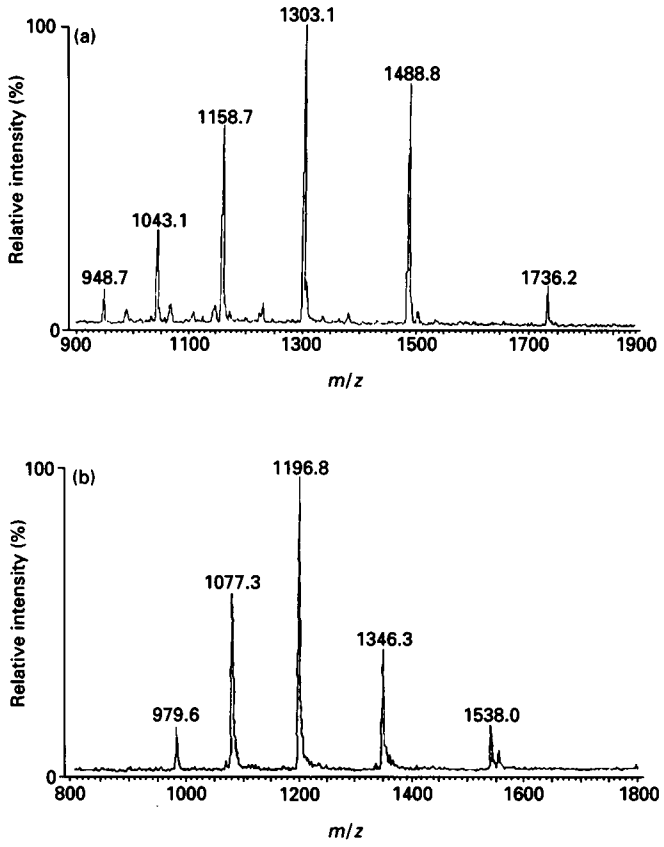

Figure 7 Electrospray mass spectra of *apo*- and *holo*-ACP isolated after hydrophobic-interaction chromatography on octyl-Sepharose CL-4B

(**a**) Measured, 10422.4 Da; calculated for *apo*-ACP, 10422.7 Da; (**b**) measured, 10762.8 Da; calculated for *holo*-ACP, 10762.0 Da.

the initial washing of the column with buffer A (90–110 ml); the *holo*-ACP eluted shortly afterwards (120–145 ml). The *apo*-ACP was not observed to elute at all with buffer A. Application of a linear gradient of propan-2-ol (0–30 %, v/v, in buffer A) resulted in elution of the *apo*-ACP (eluting at 8–12 % propan-2-ol). SDS/PAGE could not distinguish between *holo*- and *apo*-ACP; therefore, the fractions were analysed by non-denaturing PAGE (see Figures 5 and 6). Confirmation of the identities of the two species was obtained by using e.s.m.s. (see Figure 7). The spectrum in Figure 7(a) corresponds with *apo*-ACP (measured, 10422.4 Da; calculated, 10422.7 Da) and represents the material that eluted later from the octyl-Sepharose column (the higher band in non-denaturing PAGE); the material eluting earlier, corresponding with the spectrum in Figure 7(b), was found to be *holo*-ACP (measured, 10762.8 Da; calculated, 10762.0 Da). In agreement with the findings of Rock and Garwin (1979), the *holo*-ACP was not observed to adsorb to octyl-Sepharose, even when high concentrations of lyotropic salts [3.2 M $(NH_4)_2SO_4$ or 2 M KCl] were added to buffer A. Addition of these salts did, however, cause the contaminant protein to bind more strongly and caused it to elute with the *holo*-ACP. Therefore, no salt was added to the buffers used for the octyl-Sepharose chromatography. The *apo*-ACP began to elute at a propan-2-ol concentration of 8 % (v/v) in buffer A. This indicates that *apo*-ACP is slightly more hydrophobic than *holo*-ACP, but not as hydrophobic as the acyl-ACP derivatives (Rock and Garwin, 1979), as expected. The separation of *S. erythraea holo*- and *apo*-ACP represents, to the best of our knowledge, the first large-scale separation of these two species. A small-scale separation of *holo*- and *apo*-ACP species (sub-m) has been reported previously (Shen et al., 1992), but the authors did not determine at which stage in the purification, either after Mono-Q anion-exchange chromatography or after phenyl-Superose chromatography, the separation had occurred. Our findings suggest that the separation was achieved during the latter step. In our work, phenyl-Sepharose chromatography was tried as a means to separate the *S. erythraea holo*- and *apo*-ACP, but it was found that the medium was not hydrophobic enough to permit effective separation; most of the *apo*- and *holo*-ACP eluted together.

The *S. erythraea holo*-ACP isolated as described above was fully active in supporting the synthesis of acyl-ACP by extracts of *S. erythraea* using the assay developed by Hale et al. (1987). The *apo*-ACP was completely inactive. The large amounts of pure *S. erythraea apo*- and *holo*-ACP now available have permitted us to begin n.m.r. and other structural studies on these two species. In addition, the purified *apo*-ACP may be useful as a substrate in the detection of the *S. erythraea holo*-ACP synthetases that catalyse the transfer of 4′-phosphopantetheine from coenzyme A to, respectively, the *apo*-ACP that is involved in fatty-acid biosynthesis in *S. erythraea*, and the *apo*-ACP domains from the *S. erythraea* polyketide synthase that are responsible for the production of 6-deoxyerythronolide B, the initial polyketide precursor of the antibiotic erythromycin (Elovson and Vagelos, 1968; Cortes et al., 1990; Donadio et al., 1991; Staunton, 1991; Bevitt et al., 1992; Donadio and Katz, 1992; Roberts et al., 1993).

We are grateful to Dr. J. McCormick for carrying out the N-terminal sequence analysis, Dr. J. E. Cronan, Jr., for the *E. coli* strain SJ16 and Dr. S. Tabor for the pT7-7 system. This work was supported by grants from the Science and Engineering Research Council (U.K.) and the Natural Sciences and Engineering Research Council of Canada. J.S. and P.F..L. are members of the Cambridge Centre for Molecular Recognition.

REFERENCES

Bayan, N. and Therisod, H. (1989) FEBS Lett. **253**, 221–225
Bevitt, D. J., Cortes, J., Haydock, S. F. and Leadlay, P. F. (1992) Eur. J. Biochem. **204**, 39–49
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Bridges, A. M., Leadlay, P. F., Revill, W. P. and Staunton, J. (1991) J. Chem. Soc. Chem. Commun. 776–777
Brody, S. and Mikolajczyk, S. (1988) Eur. J. Biochem. **173**, 353–359
Brozek, K. A. and Raetz, C. R. H. (1990) J. Biol. Chem. **265**, 15410–15417
Butt, A. D. and Ohlrogge, J. B. (1991) Plant Physiol. **96**, 937–942
Caffrey, P., Green, B., Packman, L. C., Rawlings, B. J., Staunton, J. and Leadlay, P. F. (1991) Eur. J. Biochem. **195**, 823–830
Cortes, J., Haydock, S. F., Roberts, G. A., Bevitt, D. J. and Leadlay, P. F. (1990) Nature (London) **348**, 176–178
Cronan, J. E., Jr. (1980) J. Bacteriol. **141**, 1291–1297
Donadio, S. and Katz, L. (1992) Gene **111**, 51–60
Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J. and Katz, L. (1991) Science **252**, 675–679
Dunn, J. J. and Studier, F. W. (1983) J. Mol. Biol. **166**, 477–535
Elovson, J. and Vagelos, P. R. (1968) J. Biol. Chem. **243**, 3603–3611
Guerra, D. J., Dziewanowska, K., Ohlrogge, J. B. and Beremand, P. D. (1988) J. Biol. Chem. **263**, 4386–4391
Hale, R. S., Jordan, K. N. and Leadlay, P. F. (1987) FEBS Lett. **224**, 133–136
Hanahan, D. (1983) J. Mol. Biol. **166**, 557–580
Holak, T. A., Frederick, A. F. and Prestegard, J. H. (1987) J. Biol. Chem. **262**, 3685–3689
Hopwood, D. A. and Sherman, D. H. (1990) Annu. Rev. Genet. **24**, 37–66
Issartel, J.-P., Koronakis, V. and Hughes, C. (1991) Nature (London) **351**, 759–761
Jackowski, S. and Rock, C. O. (1981) J. Bacteriol. **148**, 926–932
Jackowski, S. and Rock, C. O. (1983) J. Biol. Chem. **258**, 15186–15191
Jacobson, G. R. and Rosenbusch, J. P. (1976) Nature (London) **261**, 23–26
Jacobson, G. R., Takacs, B. J. and Rosenbusch, J. P. (1976) Biochemistry **26**, 2297–2303

Joseph-Liauzun, E., Leplatois, P., Legoux, R., Guerveno, V., Marchese, E. and Ferrara, P. (1990) Gene **86**, 291–295
Kim, Y. and Prestegard, J. H. (1990) Proteins: Struct. Funct. Genet. **8**, 377–385
Laemmli, U. K. (1970) Nature (London) **227**, 680–686
LaVallie, E. R., DiBlasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F. and McCoy, J. M. (1993) Bio/Technology **11**, 187–193
Loo, J. A., Usdeth, J. R. and Smith, R. D. (1989) Anal. Biochem. **179**, 404–412
Lunn, C. A. and Pigiet, V. P. (1982) J. Biol. Chem. **257**, 11424–11430
Matsudaira, P. (1987) J. Biol. Chem. **262**, 10035–10038
Nossal, N. G. and Heppel, L. A. (1966) J. Biol. Chem. **241**, 3055–3062
Revill, W. P. and Leadlay, P. F. (1991) J. Bacteriol. **173**, 4379–4385
Roberts, G. A., Staunton, J. and Leadlay, P. F. (1993) Eur. J. Biochem. **214**, 305–311
Rock, C. O. and Cronan, J. E., Jr. (1979) J. Biol. Chem. **254**, 9778–9785
Rock, C. O. and Cronan, J. E., Jr. (1981) Methods Enzymol. **71**, 341–351
Rock, C. O. and Garwin, J. L. (1979) J. Biol. Chem. **254**, 7123–7128
Rock, C. O. and Jackowski, S. (1982) J. Biol. Chem. **257**, 10759–10765
Runswick, M. J., Fearnley, I. M., Skehel, J. M. and Walker, J. E. (1991) FEBS Lett. **286**, 121–124
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
Shen, B., Summers, R. G., Gramajo, H., Bibb, M. J. and Hutchinson, C. R. (1992) J. Bacteriol. **174**, 3818–3821
Staunton, J. (1991) Angew. Chem. Int. Ed. Engl. **30**, 1302–1306
Swan, D. G., Cortes, J., Hale, R. S. and Leadlay, P. F. (1989) J. Bacteriol. **171**, 5614–5619
Tabor, S. and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1074–1078
Tabor, S., Huber, H. E. and Richardson, C. C. (1987) J. Biol. Chem. **262**, 16212–16223
Therisod, H. and Kennedy, E. P. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8235–8238
Van den Boom, T. and Cronan, J. E., Jr. (1989) Annu. Rev. Microbiol. **43**, 317–343
Van der Bosch, H., Williamson, J. R. and Vagelos, P. R. (1970) Nature (London) **228**, 338–341
Zensen, R., Husmann, H., Schneider, R., Peine, T. and Weiss, H. (1992) FEBS Lett. **310**, 179–181

Received 23 March 1993; accepted 13 April 1993