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

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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-[1-14C]Alanine (53 mCi/mmol) was supplied by DuPont. [2-14C]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,
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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 Leadley, 1991). E. coli SJ16 panD22:adj-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 analysing 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 Cooamassie Brilliant Blue R250. 3C-labelised 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 Matsudaia (1987). Sequences were determined using an Applied Biosystems model 477A puls-child 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 CaCl₂ 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 (D₅₀₀, 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 x 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 x 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 x 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 electricstatic 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/H₂O (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⁺, Mg²⁺ or Ca²⁺), 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-[1-14C]-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-[1-14C]-alanine. Cultures were heat-induced at 42 °C for 30 min and then incubated for 60 min at 30 °C. Cells 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 (pGPl-2, pFEX-1) and of *E. coli* SJ16 (pGPl-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-[1-14C]-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-[1-14C]-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 (pGPl-2) was first checked for correct post-translational processing by introducing the plasmids pGPl-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-[1-14C]-alanine. The cells were heat-induced following the protocol described in this paper for *E. coli* K38 (pGPl-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;
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 (pGPI-2, pFEX-1) and E. coli SJ16 (pGPI-2, pT7-7) cultures that had been labelled with β-[1-14C]glutamine were subjected to the freeze–thaw procedure. 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 (pGPI-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 2. SDS/24% PAGE analysis of E. coli K38 (pGPI-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, holoACP; C, holo-ACP dimer; D, holoACP 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
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, 10422.4 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%, v/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 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, 11008.2 Da; calculated for holo-ACP glutathione adduct, 11076.7 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, 10764.2 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

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.
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 (NH₄)₂SO₄ 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-deoxerythronolide 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).

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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.

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