

# A small, discrete acyl carrier protein is involved in de novo fatty acid biosynthesis in *Streptomyces erythraeus*

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A heat-stable factor, required for de novo synthesis of fatty acids in the erythromycin-producing organism *Streptomyces erythraeus*, has been purified to homogeneity and identified as an acyl carrier protein (ACP). We conclude that, contrary to previous belief, fatty acid synthase in *S. erythraeus* more closely resembles the dissociable complex of *E. coli* than the tightly associated, multifunctional enzyme complex found in the related actinomycete *Mycobacterium smegmatis*.

Fatty acid synthase; Acyl carrier protein; (Streptomycetes)

## 1. INTRODUCTION

It is widely accepted that the initial stage in the biosynthesis of oligoketides, from simple precursors such as acetate, propionate and butyrate, bears a strong resemblance to the biosynthesis of fatty acids. Fatty acid synthase in streptomycetes and other actinomycetes has been assumed [1,2] to be a tightly associated multi-enzyme complex, on the same pattern as the enzyme from *Mycobacterium smegmatis*, which has a subunit structure  $\alpha_6-\beta_8$  and a total  $M_r$  of  $2 \times 10^6$  [3]. Previous work on the fatty acid synthases from *Streptomyces erythraeus* [1] and from *S. coelicolor* [4] has indicated that this might be true, but in neither case was the synthase purified to homogeneity. We wish to explore the details of the relationship between fatty acid and oligoketide biosynthesis, and we have therefore re-investigated fatty acid biosynthesis in the erythromycin producer *S. erythraeus*. In contrast to previous work, we have found that de novo fatty acid biosynthesis

depends upon the presence of a heat-stable factor which we have identified as an authentic, discrete acyl carrier protein (ACP).

## 2. MATERIALS AND METHODS

### 2.1. Materials

Methyl esters of standard branched-chain fatty acids were from Supelco. *E. coli* acyl carrier protein and CoASH were from Sigma. Isobutyryl-CoA was synthesized from the corresponding anhydride. *Propionibacterium shermanii* was grown as in [5], and ACP [6] was purified from it by the method described in this paper for *S. erythraeus* ACP. [2- $^{14}$ C]Malonyl-CoA (55 Ci/mol) was from Amersham International. SDS-PAGE was carried out as in [7].

*S. erythraeus* NRRL 2338 (recently re-classified as *Saccharopolyspora erythraea* [8]) was grown as in [9]. After 36 h growth, the mycelium was harvested, washed twice with cold sucrose (10%, v/v), and stored at  $-20^\circ\text{C}$ . The cell paste was resuspended in an equal volume of ice-cold 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF and 10% (v/v) glycerol. The cells (100 g) were broken by a single pass through a bead mill (KDL

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DynoMill, Willy Bachofen, Zürich) and the extracts were clarified by centrifugation at  $30000 \times g$  for 15 min. Streptomycin sulphate was added with stirring to a final concentration of 1% (w/v), and the resulting precipitate was removed by centrifugation as before. Solid ammonium sulphate (39.8 g/100 ml) was added, and after 30 min at 4°C the precipitated protein was collected by centrifugation as before. This pellet was suspended in 40 ml of the buffer used in breaking the cells, and after dialysis against the same buffer was designated fraction A by analogy with similar work using *E. coli* [10]. To the supernatant, more ammonium sulphate was added (16.8 g/100 ml) and the pH was adjusted to 4.0 with glacial acetic acid. The precipitated protein was collected by prolonged (usually 1 h) centrifugation at  $30000 \times g$ , suspended in 8 ml of the original buffer, and after dialysis against this buffer it was designated the supernatant fraction. In preliminary experiments a heat-stable factor was obtained by heating cell-free extracts for 15 min at 80°C and removing the precipitate by centrifugation. Cell-free extracts of *S. coelicolor* A3(2) were obtained by an identical procedure.

### 2.2. Standard fatty acid synthase assay

Each incubation contained, at 30°C in a final volume of 0.5 ml, 100 mM potassium phosphate buffer, pH 7.0, 1 mM DTT, 0.1 mM NADH, 0.1 mM NADPH, 0.08 mM isobutyryl-CoA, 50  $\mu$ l fraction A and up to 0.2 ml supernatant fraction or ACP. Reaction was initiated with 1  $\mu$ l (20 nCi) [2-<sup>14</sup>C]malonyl-CoA. After 30 min, 50  $\mu$ l isostearic acid (Sigma) was added as a carrier, and the reaction was stopped with 100  $\mu$ l of 60% KOH. The mixture was boiled for 30 min at 100°C, cooled, and 110  $\mu$ l MeOH and 250  $\mu$ l of 6 M HCl were added. The mixture was extracted twice with 0.5-ml portions of heptane (HPLC grade, Aldrich), and the organic layer was washed once with 0.5 ml saturated NaCl solution. Portions of the heptane layer were mixed with 10 vols of Optiphase scintillant (LKB) and counted for radioactivity.

### 2.3. Purification of ACP from *S. erythraeus*

8 ml of undialyzed supernatant fraction (see above) was applied to a Sephadex G-75 (Pharmacia) column (5  $\times$  80 cm) equilibrated with

50 mM Tris-Cl buffer, pH 8.0, containing 1 mM EDTA. Active fractions were combined (250 ml). A portion (50 ml) was applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated in 50 mM Tris-Cl, pH 8.0. The ACP was eluted in fractions of 1 ml at 1 ml/min, using a gradient of 0–1 M KCl over 20 min, and was collected essentially in a single fraction eluting at about 0.4 M KCl. Final purification before N-terminal sequence analysis was on a Brownlee RP-300 C-8 reverse-phase column (3 cm  $\times$  2 mm) fitted with a Newguard guard column. The gradient used for elution was 0–75% CH<sub>3</sub>CN in 10 mM ammonium acetate, pH 5.8. Automated N-terminal sequence analysis was done using an Applied Biosystems 470A gas-phase sequencer.

## 3. RESULTS AND DISCUSSION

In preliminary experiments it was noted that fatty acid biosynthesis in extracts from *S. erythraeus* required both a fraction A and a heat-stable factor (fig.1). The supernatant fraction from the ammonium sulphate treatment contained this factor, and it was purified to homogeneity using as an assay the ability to restore fatty acid synthase activity to fraction A. A particularly revealing finding was that authentic *E. coli* ACP could substitute for the heat-stable factor in this assay (fig.2). Fatty acid biosynthesis by an entirely analogous fraction A from *S. coelicolor* could be

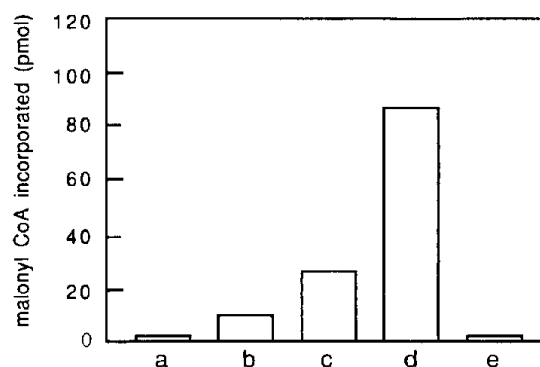


Fig.1. Effect of added heat-stable factor on fatty acid synthesis by *S. erythraeus* fraction A: (a) no protein; (b) 20  $\mu$ l fraction A only; (c) 20  $\mu$ l fraction A and 20  $\mu$ l factor; (d) 100  $\mu$ l fraction A and 50  $\mu$ l factor; (e) 20  $\mu$ l factor only.

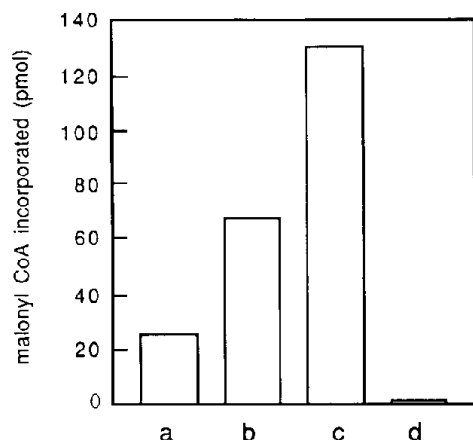


Fig.2. Effect of *E. coli* ACP on fatty acid synthesis by *S. erythraeus* fraction A: incubations (a–c) contained 50 µl fraction A and (a) no additions; (b) 30 µl heat-stable factor; (c) 30 µl (30 µg) *E. coli* ACP; (d) contained no protein.

stimulated by its own supernatant fraction, by that from *S. erythraeus* and by purified, authentic ACP from *P. shermanii* (not shown). It therefore seems likely that other streptomycetes also contain ACPs of this type.

The purified ACP from *S. erythraeus* has an apparent  $M_r$  of 20000, as judged by the position of its elution on a calibrated TSK 2000SWG gel filtration column (Anachem, Luton, England). This is similar to results obtained with *E. coli* ACP, even though the latter protein is known to contain only 77 amino acids and to be a monomer in solution of  $M_r$  8847. The *S. erythraeus* protein has an apparent  $M_r$  of 6000 in SDS-PAGE. ACPs from a variety of bacterial sources behave anomalously (for reasons which are poorly understood) on gel filtration and in SDS-PAGE [11,12], and the true  $M_r$  will only be known when the complete amino acid sequence is available.

The acyl chain length was analysed by radio GLC of the reaction products (as their methyl esters). The major product under the standard

assay conditions showed a retention time appropriate for a terminally branched  $C_8$  fatty acid methyl ester (not shown). The predominant fatty acid found in *S. erythraeus* lipids is iso- $C_{16}:0$  (Embley, M., personal communication) and the shorter chains found here are presumably the result of a particular choice of concentrations for acyl-CoA substrates and ACP in the assay [13].

Fig.3 shows a comparison of the N-terminal amino acid sequence of the *S. erythraeus* ACP with the corresponding sequence from *E. coli* [14]. The evident and extensive homology confirms the identity of the purified protein as an ACP. No evidence was obtained for the presence of a 'ragged' N-terminus, which might have been expected if this ACP were the product of adventitious proteolysis of an ACP domain. The 4'-phosphopantetheine attachment site in *E. coli* ACP is Ser-36, and the corresponding site in the *S. erythraeus* ACP is inferred to be Ser-39 (fig.3). The sequence in this region is highly conserved between the two species, and the serine residue in each case is the fourth residue in a predicted  $\beta$ -bend [11].

These findings raise interesting questions about the relationship between fatty acid and oligoketide biosynthesis in this organism. It is possible, for example, that the two processes share the same pool of ACP, while other enzyme components are distinct. The recognition that fatty acid (and possibly oligoketide) biosynthesis may involve a dissociable complex will now facilitate the design of specific assays for each functional component. In addition, the availability of amino acid sequence information for an authentic ACP will allow the construction of oligonucleotide probes for the corresponding structural gene(s) which should be more effective than probes based on 'consensus' sequences [15].

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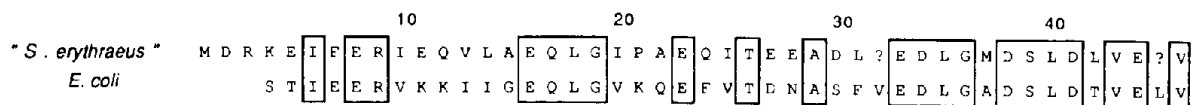


Fig.3. Alignment of the N-terminal amino acid sequence of *S. erythraeus* ACP with that of *E. coli* ACP.

measurements, and Dr L.C. Packman for N-terminal sequence analysis. This work was supported by a project grant from the Medical Research Council.

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