Use of Electrospray Mass Spectrometry in Enzymic Studies on Acyl Carrier Protein Implicated in Fatty Acid Biosynthesis in *Saccharopolyspora erythraea*

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Two types of experiments are used to confirm that an acyl carrier protein (ACP) is involved in fatty acid biosynthesis using a cell-free extract of *Saccharopolyspora erythraea*: turnover using a chemically prepared acyl derivative of the ACP, and isolation of acylated intermediates produced by the action of the fatty acid synthase were detected by electrospray mass spectrometry.

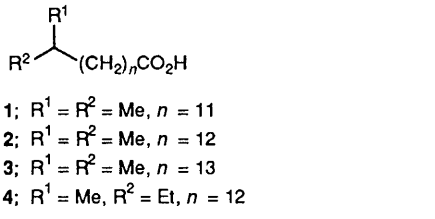
In the previous paper we described an investigation of the chemical reactivity of an acyl carrier protein (ACP) from *Saccharopolyspora erythraea* using electrospray mass spectrometry (ESMS).[1] Here we demonstrate the application of the technique to an investigation of the role of this ACP in fatty acid biosynthesis.[2]

Our first experiments involved enzymic studies with an acylated ACP, prepared by chemical methods. In previous work, the free ACP was added to a cell-free extract of *S. erythraea* (depleted in endogenous ACP) in the presence of radiolabelled malonyl coenzyme A (CoA) and the CoA derivative of a branched-chain starter acid such as isobutyric acid or isovaleric acid. Such incubations produce radiolabelled acyl-ACP derivatives, presumably as a mixture of species differing in the length and type of attached acyl chain.[3] Although the exact nature of the products has not been established, it is inferred that this enzymic process is responsible for production of the predominant fatty acyl constituents, **1–4**, of *S. erythraea* lipids.[4]

We have now obtained direct evidence that an acyl group attached on the ACP can be chain extended in this cell-free system by using chemically acylated ACP in the assay. A C_8-derivative (7-methylheptanoyl) was selected for this initial study on the grounds that it marks the approximate half-way point of the chain extension process. Any evidence for further extension(s) would therefore establish the competence of the ACP in the production of longer-chain fatty acids. Following the procedure described in the previous paper, 7-methylheptanoic acid as its imidazolium derivative was used to acylate the ACP.† The purity of the acylated protein was checked by ESMS. It consisted, as expected, of unmodified *apo*-ACP and acylated *holo*-ACP. The acylation mixture was allowed to stand for 2 h to ensure that any excess acyl imidazole had undergone hydrolysis and would not interfere in the cell-free system.

The sample of acyl ACP was then added to the cell-free extract of *S. erythraea* without any additional source of starter acyl group. After a suitable incubation period, the ACP fraction was recovered and shown to be radioactive. The relative efficiencies of the C_8-acyl ACP and a normal starter acyl species, isovaleryl CoA, in inducing incorporation of radioactivity from [2-^{14}C]malonate were assessed by running parallel incubations under equivalent conditions. The acyl ACP proved to be significantly more efficient (1.4-fold) even at lower concentration (4.7 *vs.* 25 μmol dm^{-3}). In a control experiment it was established that turnover in an incubation mixture containing the acyl-ACP did not require the presence of added free ACP, unlike one supplied with the CoA ester as source of the starter unit. The effectiveness of the synthetic acyl ACP in the assay confirms that the structural integrity of the protein is maintained under the conditions employed for chemical acylation. The way is therefore clear for a systematic study of the acylated ACP's produced by the chain extension process *in vitro*.

In preliminary experiments we have used ESMS to analyse the composition of the mixtures of acyl ACP's produced in an incubation supplied with free ACP and isovaleryl CoA as the source of starter acyl units. Chain extension in this case would be expected to lead to a series of fatty acyl ACP derivatives with odd numbers of carbons, up to a maximum of seventeen. After incubation, the ACP fraction was recovered from the incubation mixture by selective precipitation with a mixture of $CHCl_3$ and MeOH, (adapted from the method of Bligh and Dyer[6]) and analysed directly by ESMS. As expected, the series of four peaks (marked *A*), derived from the *apo*-ACP, remained intense (see Fig. 1). Significantly, however, the four peaks in the series of the *holo*-ACP (*B*) were considerably reduced in intensity from the levels seen for the original ACP mixture, and instead there were several series of peaks corresponding in mass to various acylated *holo*-ACP derivatives, in each case at slightly higher mass values than the



1; $R^1 = R^2 = Me$, $n = 11$
2; $R^1 = R^2 = Me$, $n = 12$
3; $R^1 = R^2 = Me$, $n = 13$
4; $R^1 = Me$, $R^2 = Et$, $n = 12$

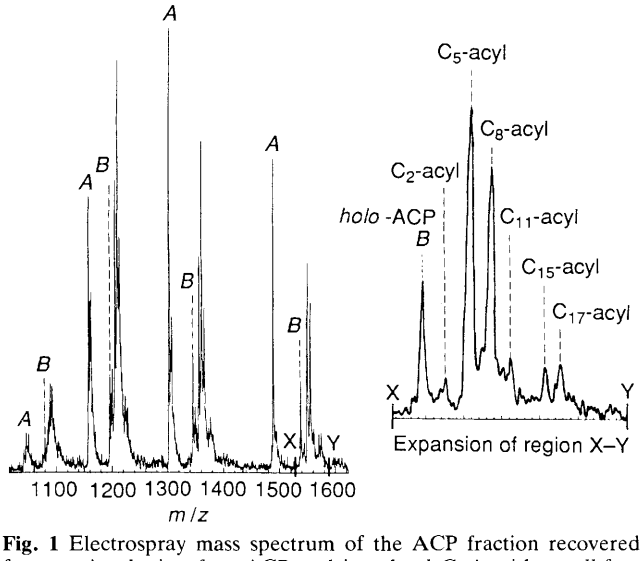


Fig. 1 Electrospray mass spectrum of the ACP fraction recovered from an incubation free ACP and isovaleryl CoA with a cell-free system from *S. erythraea*

† The ACP used in all the experiments described in this paper was produced by over-expression in *Escherichia coli*.[5] It was contaminated with a related protein, the *apo*-ACP, which lacked the prosthetic group (4′-phosphopantetheine). This contaminant (up to 50% of total protein) was inert in the assays and did not interfere in the ESMS analysis.

Table 1 ESMS analysis of a mixture of fatty acyl derivatives of the *holo*-ACP produced from the free ACP on incubation with a cell-free system from *S. erythraea* containing added isovaleryl CoA

Component	Molecular Weight		
	Observed	σ[a]	Calculated[b]
Apo-ACP	10423	1	10423
Holo-ACP	10763	1	10763
C_2-Acyl ACP	10806	4	10807
C_5-Acyl ACP	10851	3	10849
C_8-Acyl ACP	10890	1	10891
C_{11}-Acyl ACP	10929	1	10933
C_{15}-Acyl ACP	10988	2	10989
C_{17}-Acyl ACP	11017	6	11017

[a] Standard deviations. [b] Molecular weights are calculated using the average isotopic masses weighted by abundance, *e.g.* C = 12.011, H = 1.008 and N = 14.007. This corresponds to the centroid of the molecular ion distribution.

holo-ACP, as would be expected. In an expansion of one of the four clusters of peaks shown in Fig. 1, six individual peaks are assigned to acyl ACP components on the basis of the mass measurements presented below.

From the observed masses listed in Table 1 it is clear that four of the new series of peaks correspond satisfactorily with calculated mass values for *holo*-ACP's acylated with fatty acyl chains having odd numbers of carbons, and differing in mass from the added starter acyl residue by multiples of 28 units, the mass of two methylene groups: a C_5-acyl ACP, and C_{11}-, C_{15}-, and C_{17}-analogues, the latter corresponding to the expected maximum chain length for fatty acids in *S. erythraea*. Surprisingly, there are, in addition, two relatively intense series of peaks corresponding to added acyl groups with even numbers of carbons (C_2 and C_8). It has been shown that acetyl CoA does not stimulate the uptake of radiolabelled malonate in the cell-free system.[7] However, acetyl-*holo*-ACP could be generated *in situ* by decarboxylation of malonyl-*holo*-ACP, and the resulting acetyl group transferred adventitiously to the ketoacyl synthase by the normal mechanism for transferring acyl groups from the ACP to the ketoacyl synthase at the end of successive cycles of chain extension. Normal chain extension would then lead to a succession of acyl ACP intermediates with acyl chains containing even numbers of carbons. The fact that this series appears to accumulate relatively large amounts of the C_8-intermediate may reflect the relative inefficiency of the next chain extension cycle in the case of straight-chain intermediates.

Further work is in progress to confirm the identity of the acyl chains which accumulate on the *holo*-ACP. For example, the resolution of our existing instrument does not allow us to distinguish directly between saturated acyl chains and other intermediates which might reasonably accumulate, depending on which step in the fatty acid biosynthetic cycle is rate-limiting. However, it is clear that the added *holo*-ACP has been converted to a mixture of acyl *holo*-ACP derivatives including the one corresponding to the added C_5-starter acyl group, and that this has been used for chain assembly, by addition of successive C_2-units, up to a maximum of six, the normal limit of chain extension in *S. erythraea*.

ESMS has already been used to observe previously well-characterised covalent enzyme-bound intermediates,[8] to monitor post-translational modification of proteins[5] and to analyse active site-directed inhibition of an enzyme.[9] Our present results demonstrate the potential of ESMS for direct observation, even in cell-free extracts, of sequential biosynthetic reactions which take place with the substrates covalently bound to proteins, without release of free intermediates. This approach should find wide application in the study of such assembly processes on, *inter alia*, polyketide synthases and non-ribosomal peptide synthases.

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