Use of Electrospray Mass Spectrometry to assay Chemical Reactivity of an Acyl Carrier Protein involved in Fatty Acid Biosynthesis in *Saccharopolyspora erythraea*

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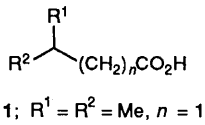
Electrospray mass spectrometry (ESMS) is used to monitor the chemical reactivity of the thiol-containing 4′-phosphopantetheine residue of an acyl carrier protein implicated in fatty acid biosynthesis in *Saccharopolyspora erythraea*.

Electrospray mass spectrometry (ESMS) is attracting increasing interest as a rapid and convenient technique to determine the mass of protein molecules with a high degree of precision.[1] In this paper we describe exploratory experiments which lay the foundation for its use in studying the sequential steps of a biosynthetic pathway in which the intermediates remain bound to the proteins of the biosynthetic apparatus and are therefore difficult to investigate by conventional techniques.

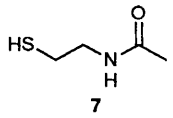
The system under investigation is fatty acid biosynthesis in the Gram positive bacterium *Saccharopolyspora erythraea*, the organism which produces the important macrolide antibiotic erythromycin.[2] Analysis of the lipids of this organism has established that the major fatty acid component (31%) is the C_{16} compound, isopalmitic acid **1**.[3] This corresponds to the use of isobutyrate **2** as the starter acyl residue instead of acetate. Other major components (15 and 13% respectively) are the C_{15} acid **3** and the C_{17} acid **4** which would be derived from isovaleric acid **5** and anteisovaleric acid **6**, respectively.

In previous work, a discrete acyl carrier protein (ACP) has been isolated from *S. erythraea* and purified to homogeneity.[4] This ACP promotes the formation of fatty acyl ACP species in cell-free extracts of *S. erythraea* implying that fatty acid biosynthesis in this organism is patterned on the dissociable (type II) fatty acid synthases typical of bacteria.[5] The gene coding for this protein has been sequenced and also overexpressed in *Escherichia coli*.[6] The protein is now readily available from this source (more than 100 mg per 15 l of culture) and is therefore an ideal candidate for the evaluation of ESMS as an assay technique.

Our work has been carried out on a VG BioQ mass spectrometer supplied by VG Biotech. This is a quadrupole instrument capable of determining the mass of our proteins with an accuracy of the order of 1 in 10^4. The purified ACP fraction isolated from the expression system gave a satisfactory spectrum when injected as a solution in MeOH–H_2O (1 : 1) containing formic acid (10%). The spectrum shown in Fig. 1 contains two series of peaks. The first, marked *A*, corresponds to a protein of *M* 10 423 ± 1 and can be attributed to the *apo*-ACP, the unmodified product of the expression system (calc. *M* 10 423). The second series, *B*, corresponds to a protein of *M* 10 763 ± 2, which accords with the mass (10 463) calculated for protein (the *holo*-ACP) which has been post-translationally modified by addition of the prosthetic group, a 4′-phosphopantetheine unit. In both series the peak of lowest *m/z* value arises from molecules which are protonated at all ten available basic residues.



1; $R^1 = R^2 = Me$, $n = 12$
2; $R^1 = R^2 = Me$, $n = 0$
3; $R^1 = R^2 = Me$, $n = 11$
4; $R^1 = Me$, $R^2 = Et$, $n = 12$
5; $R^1 = R^2 = Me$, $n = 1$
6; $R^1 = Me$, $R^2 = Et$, $n = 0$



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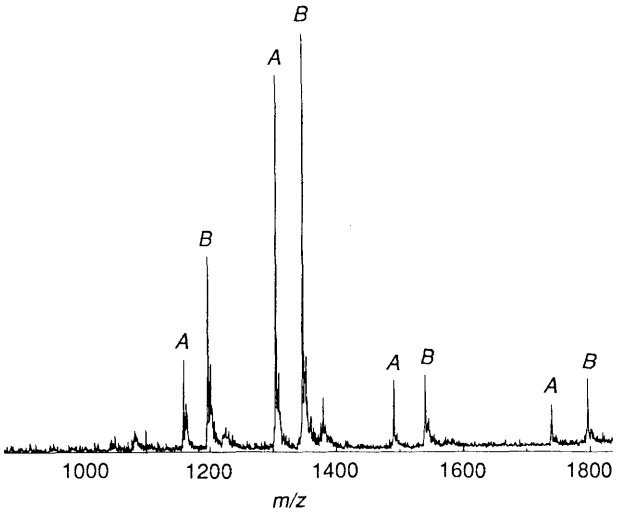

Fig. 1 Electrospray mass spectrum of the acyl carrier protein overexpressed in *E. coli*

Table 1 Analysis of acylated ACP's by ESMS

	Molecular weights of acylated ACP's		
Acyl Group	Observed	σ[a]	Calculated[b]
Acetyl	10808	3	10807
Propionyl	10820	3	10821
n-Butyryl	10835	3	10835
Isobutyryl **2**	10834	4	10835
Isovaleryl **5**	10847	2	10849
Anteisovaleryl **6**	10849	1	10849
Trimethylacetyl	10848	1	10849
4-Methylvaleryl	10861	3	10863
6-Methylheptanoyl	10888	1	10891
8-Methylnonanoyl	10917	1	10919
2-(Trifluoromethyl)propionyl	10888	2	10889

[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.

The presence of two components was confirmed by HPLC analysis of the mixture, using a reversed phase C_8-column and gradient elution with acetonitrile–water mixtures containing 0.1% trifluoroacetic acid. Two significant peaks were observed, and when the components were isolated and identified by ESMS, one proved to be, as expected, the *apo*-ACP, and the other the *holo*-ACP. In the following studies the mixture of *apo*-ACP and *holo*-ACP was used, rather than the purified *holo*-ACP, to minimise the risk of denaturation in the separation process, and also because the former component serves as a convenient control for evaluating changes in the latter in the various experiments.

During fatty acid biosynthesis the ACP carries the developing acyl chain by means of a covalent thioester link to the terminal thiol of the phosphopantetheine residue.[7] We therefore needed to develop mild chemical methods for acylating the active site thiol to produce potentially active biosynthetic intermediates for enzymic studies. Earlier work with the ACP from *E. coli* has shown that acyl imidazoles are convenient reagents.[8] This approach has also proved successful with our *holo*-ACP. In general an aqueous solution of the ACP fraction (*ca.* 100 μmol dm^{-3}), adjusted to pH 7 by treatment with ammonia solution, was treated with two equivalents of the acyl imidazole. The reactions examined are listed in Table 1. In every case the reaction of the *holo*-ACP, as monitored by ESMS, was complete within ten minutes to give a quantitative yield of a monoacyl derivative. Only the *holo*-ACP reacted under these conditions, but on prolonged treatment (24 h) with excess of reagent (10 fold) the *apo*-ACP was also converted to a monoacyl derivative. Significantly, the acylated *holo*-ACP underwent a second acylation at approximately the same rate, and presumably at the same site, which we suggest may be the *N*-terminal amino-group. This second acylation is too slow to detract from the preparative utility of the monoacylation procedure for the *holo*-ACP.

Evidence that the monoacylation of the *holo*-ACP with acyl imidazoles has occurred at the active site thiol residue was sought by investigating an alternative reaction of this group, conversion to a disulphide with an external thiolating reagent. These experiments are unambiguous because the phosphopantetheine residue carries the only free thiol group in the *holo*-ACP. Attempts to form an S–S link between the active site thiol and an *N*-acetyl cysteamine residue using the disulphide derivative of *N*-acetylcysteamine proved unsuccessful, but the *holo*-ACP did react rapidly with *p*-nitrophenyl disulphide. Only the *holo*-ACP reacted and it was converted in quantitative yield to a derivative whose molecular weight, as measured by ESMS (10919 ± 1), was consistent with that calculated for the expected ACP-SS-Ar derivative (10919).

The monoacylated *holo*-ACP produced by treatment with isobutyryl imidazole did not react with this reagent showing that acylation had occurred on the thiol group. The failure of the disulphide derivative to undergo rapid monoacylation on treatment with isobutyryl imidazole provided further evidence that it is the active site thiol of the free *holo*-ACP which undergoes this reaction.

Attempts were also made to acylate the *holo*-ACP with *S*-acyl derivatives of *N*-acetylcysteamine **7**. On mixing with the ACP solution under the standard conditions used in the experiments with acyl imidazoles no reaction was detected even after 24 h. These acyl coenzyme A (CoA) mimics are therefore not sufficiently reactive for the chemical acylation of the *halo*-ACP, although they may be effective substrates for acyl transfer in the presence of the acyl transferase which carries out this reaction *in vivo* using the equivalent CoA derivatives.

Experiments using ESMS were also carried out to assess the stability of the acylated *holo*-ACP derivatives listed in Table 1. All were stable for five days in aqueous solution at pH 7. This demonstration that the acylated *holo*-ACP's are stable at physiological pH is vitally important for the various biochemical studies described in the next paper. In a study of the effect of alkali, the isobutyryl derivative was found to undergo rapid deacylation at pH 9 to produce the free *holo*-ACP.

ESMS is, therefore, an extremely convenient tool for monitoring chemical reactions of our ACP in various types of experiment. In the following paper we demonstrate the utility of the technique in studies of related enzymic transformations.

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