Investigations into the post-translational modification and mechanism of isopenicillin N:acyl-CoA acyltransferase using electrospray mass spectrometry

Robin T. APLIN, Jack E. BALDWIN*, Peter L. ROACH, Carol V. ROBINSON and Christopher J. SCHOFIELD

The Dyson Perrins Laboratory and Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, U.K.

Electrospray mass spectrometry (e.s.m.s.) was used to confirm the position of the post-translational cleavage of the isopenicillin N:acyl-CoA acyltransferase preprotein to give the α - and β -subunits. The e.s.m.s. studies suggested partial modification of the α -subunit *in vivo* by exogenously added substituted acetic acids. E.s.m.s. has also allowed the observation *in vitro* of the

transfer of the acyl group from several acyl-CoAs to the β -subunit. N.m.r. data for the CoA species have been deposited as Supplementary Publication SUP 500173 (2 pages) at the British Library Document Supply Centre (DSC), Boston Spa, Wetherby, West Yorkshire LS23 7BQ, from whom copies can be obtained on the terms indicated in Biochem. J. (1993) **289**, 9.

INTRODUCTION

The final step in the production of penicillins with hydrophobic side chains in *Penicillium chrysogenum* is carried out by isopenicillin N:acyl-CoA acyltransferase (AT). This enzyme removes the α -amino adipoyl group of isopenicillin N (IPN) and replaces it with an acyl group from an acyl-CoA (Scheme 1) [1,2].

Alvarez et al. [3] reported the purification of AT to apparent homogeneity, as a monomeric protein with a molecular mass of 29 kDa. Subsequently, the enzyme was purified as an α , β -heterodimer requiring both 29 (β -) and 11 (α -) kDa subunits for activity [4], and the amino acid sequence data obtained allowed the cloning of the *penDE* gene [5–6a]. The gene sequence showed that the α - and β -subunits were derived from a single 40 kDa preprotein, although different cleavage sites were proposed by Barredo et al. [5] (Asp¹⁰¹-Gly¹⁰² or Cys¹⁰³-Thr¹⁰⁴) and Tobin et al. [6,6a] (Gly¹⁰²-Cys¹⁰³). The deduced protein sequence of AT was proposed to contain a potential acylation site at residues 307–311 in the preprotein which is similar in sequence (-GXSG-) to those identified in the thioesterase domain of fatty acid synthases [7]. To date no studies on the mechanism of the enzyme have been reported.

Recently, electrospray mass spectrometry (e.s.m.s.) has been shown to be a potentially powerful technique for the study of enzyme mechanisms and post-translational modifications. For example, e.s.m.s. has been used to investigate the role of acyl enzyme intermediates in the cases of the acyl carrier protein involved in fatty acid biosynthesis [8], the acylenzyme intermediate of β -lactamases [9] and the acylenzyme inhibitor-substrate complexes of the serine proteases [10,11]. In this paper, we report the use of e.s.m.s. to investigate the mechanism and nature of the post-translational modifications of AT.

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EXPERIMENTAL

Materials

Superdex 200 Prep Grade 35/600 and Mono Q 5/5 anion-exchange and Sephadex PD10 G-25M columns and Sephadex G-50 were obtained from Pharmacia Milton Keynes, Bucks., U.K. Affi-gel Blue was obtained from Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K. Molecular-mass markers were obtained from BDH Chemicals, Poole, Dorset, U.K. Centricon 10 concentrators were obtained from Amicon, Stonehouse, Gloucestershire, U.K. Phenylacetyl-, nonanoyl-, octanoyl-, heptanoyl-, hexanoyl- and pentanoyl-CoAs, dithiothreitol, 4-bromophenylacetic acid, phenylacetic acid, elastase and (NH₄)₂SO₄ were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Ammonium formate and 1,1'-carbonyl diimidazole were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Methanol and acetonitrile (h.p.l.c. grade) were obtained from Rathburn Chemicals, Walkerburn, Scotland, U.K. 6-Aminopenicillanic acid (6-APA) was the gift of Eli Lilly and Co., Indianapolis, IN, U.S.A. H.p.l.c. equipment was from Millipore (U.K.) Ltd., Northwich, Cheshire, U.K. Filtration units (0.2 μ m) were obtained from Sartorius G.m.b.H., Gottingen, Germany.

General methods

SDS/PAGE was conducted by the method of Laemmli [12], with 3% acrylamide in the stacking gel and 16.5% acrylamide in the separating gel. Protein concentrations were determined by the method of Bradford [13] using BSA as the standard. Semi-preparative h.p.l.c. was performed using a Waters 600E system controller, 600 pumps and 490E detector. F.p.l.c. was performed using a Pharmacia LCC-500 system controller and either P-500 or P-6000 dual pumps. ¹H-n.m.r. spectra were recorded on a Bruker AM500 spectrometer operating at 500.13 MHz and referenced to the residual ammonium formate buffer signal (8.46 p.p.m.).

Scheme 1 Biosynthesis of penicillin G from IPN

AT catalyses both steps (i) and (ii) [1,6].

Organisms and culture conditions

P. chrysogenum (S.C. 6140, A.T.C.C. 2044) was grown as described previously [2,4]. The growth medium was supplemented for the production of hydrophobic penicillins by the addition of sodium phenoxyacetate (6 g/l), sodium phenylacetate (40 mg/l), sodium 4-bromophenylacetate (200 mg/l) or growth without further additions as required. The mycelia were harvested after 48 h growth in a 30-litre fermenter, filtered, washed twice with water, once with 1% (w/v) NaCl and stored at -80°C .

Enzyme assay

Enzyme activity was measured by the formation of penicillin G. The final assay mixture contained 0.4 mM 6-APA, 1 mM phenylacetyl-CoA, 5 mM dithiothreitol and 50 mM Tris/HCl, pH 8.0, and an appropriate amount of the enzyme in a total volume of 30 μl . After incubation at 27°C for 15 min the reaction was stopped by the addition of 30 μl of ice-cold methanol and shaken vigorously. The precipitated proteins were pelleted by centrifugation and a 50 μl portion was plated into wells made in

Staphylococcus aureus N.C.T.C. 6571 agar diffusion bioassay plates [6].

Preparation of acyl-CoA thioesters

Commercially available acyl-CoAs were further purified by reversed-phase h.p.l.c. The dual-wavelength detector was set at 220 and 254 nm. Each sample was loaded on to a Hypersil ODS reversed-phase silica column (250 mm $\times$ 10 mm internal diameter) equilibrated in 2.5% (v/v) acetonitrile in 10 mM ammonium formate, pH 5.5. After isocratic elution for 5 min, the column was developed from 2.5 to 32.5% acetonitrile over 20 min at a flow rate of 4 ml/min. Removal of the volatile h.p.l.c. solvents by lyophilization afforded the acyl-CoAs as white solids.

Pentanoyl-, hex-3-enoyl-, 4-methoxyphenylacetyl-, phenoxyacetyl- and phenylacetyl-CoAs were prepared by the general method of Kawaguchi et al. [14]. The acid (7.5 μmol) was added to a solution of 1,1'-carbonyldi-imidazole (1.21 mg, 7.5 μmol) in tetrahydrofuran (150 μl). After 30 min at room temperature, a solution of CoA (3.8 mg, 5 μmol) in water (75 μl) was added and the resulting solution mixed. After reaction for 4 h at room temperature, the reaction mixture was diluted fivefold in 10 mM ammonium formate, pH 5.5, and the acyl-CoAs were purified by reversed-phase h.p.l.c. (as above). Removal of the volatile h.p.l.c. solvents by lyophilization afforded the acyl-CoAs as white solids which were characterized by ^1H -n.m.r. and e.s.m.s.

Enzyme purification

All procedures were carried out at 4°C . *P. chrysogenum* mycelia (approx. 200 g wet weight) were resuspended in buffer A (50 mM Tris/HCl, 5 mM dithiothreitol, pH 8.0) and lysed by sonication (Heat Systems-Ultrasonics W-380 model) with a power setting of 5, for six 30 s bursts punctuated with 30 s cooling intervals. Insoluble debris was pelleted by centrifugation at 26000 g for 20 min. Poly(ethyleneimine)/HCl (5%, w/v), pH 8.0, was added to the supernatant to a final concentration of 0.1% (v/v) and stirred for 10 min. The resultant suspension was centrifuged at 26000 g for 20 min. $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant fraction to 40% saturation and stirred for 10 min. The precipitate was pelleted by centrifugation at 26000 g for 30 min and the pellet was discarded. Further $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 60% saturation and stirred for 10 min. The precipitate was recovered by centrifugation at 26000 g for 30 min.

The pellet was resuspended in the minimum volume of buffer A (5–10 ml), filtered (0.2 μm filter unit) and applied to a Superdex 200 Prep Grade 35/600 column at a flow rate of 0.5 ml/min. The flow rate was increased (to 3 ml/min) after 30 min and 3 ml fractions were collected between 240 and 640 ml and assayed for protein and enzyme activity.

The AT activity was eluted between 348 and 378 ml and these fractions were pooled and applied to an Affi-gel Blue column (90 mm $\times$ 15 mm) equilibrated with buffer A at a flow rate of 1.2 ml/min. The column was then washed with 50 ml of buffer A and the enzyme was eluted with a linear 20 ml gradient of 0–0.75 M NaCl in buffer A, and 3 ml fractions were collected and assayed for protein and enzyme activity. The AT activity was eluted between 36 and 72 ml and these fractions were pooled, desalted on a Sephadex G-50 column (40 mm $\times$ 200 mm) equilibrated with buffer A and applied to a Mono Q 5/5 anion-exchange column equilibrated with buffer A at a flow rate of 1.5 ml/min. The column was washed with 20 ml of buffer A. A 30 ml linear 0–350 mM gradient of NaCl in buffer A was applied, and 1 ml fractions were collected and assayed for activity and

Table 1 Purification of AT from *P. chrysogenum* grown on medium supplemented with sodium phenoxylacetate

Fraction	Protein (mg)	Total activity (nmol/s)	Specific activity (nmol/s per mg)	Yield (%)	Purification (fold)
Crude lysate	600.0	120.00	0.20	100.0	1.0
S200 pool (fractions 36–46)	45.0	33.75	0.75	28.1	3.8
Affi-gel Blue pool (fractions 13–24)	14.0	29.40	2.10	24.5	10.5
Mono Q 5/5 eluate					
Fraction 11	0.14	0.27	1.92	0.2	9.9
Fraction 12	0.20	0.51	2.55	0.4	12.8
Fraction 13	0.25	2.10	8.40	1.5	42.0
Fraction 14	0.21	3.54	16.86	3.0	84.3
Fraction 15	0.16	0.88	5.50	0.8	27.5

protein. Fractions shown to be at least 95 % pure by SDS/PAGE were pooled, and salt was removed using a Sephadex PD10 G-25 M column equilibrated in buffer B (20 mM Tris/HCl, 5 mM dithiothreitol, pH 8.0). The protein solution was then concentrated in a Centricon 10 microconcentrator to a concentration of 0.5–1.0 mg/ml.

E.s.m.s

Electrospray mass spectra of AT were measured on a VG BioQ triple quadrupole atmospheric pressure mass spectrometer equipped with an electrospray interface operating in positive ion mode. The mass spectrometer was scanned over the mass range 650–1550 Da. The instrument was calibrated with horse heart myoglobin (20 pmol/ μ l, molecular mass 16951.5 Da).

Initial experiments to characterize AT by e.s.m.s. used an AT solution (10–20 pmol/ μ l) in buffer B mixed with an equal volume of methanol or acetonitrile containing 2 % (v/v) acetic acid. Sample solutions used to investigate the acylation of AT or elastase by acyl-CoAs were prepared by mixing 5 μ l of the enzyme solution (10–20 pmol/ μ l) in buffer B with 1–4 μ l of the acyl-CoA solution (150 pmol/ μ l) in water. Samples were taken after 3, 6 and 12 min and mixed with an equal volume of methanol or acetonitrile and analysed immediately. The effect of adding 6-APA or β -alanine was compared by incubating AT for 3 min with heptanoyl-CoA and then adding 2 mol-equiv. of either 6-APA or β -alanine followed by incubation for a further 3 min. The reactions were stopped by the addition of acetonitrile or methanol to 50 % (v/v) and the mixtures analysed immediately by e.s.m.s.

RESULTS AND DISCUSSION

Purification of AT from *P. chrysogenum* S.C. 6140

The purification of AT was carried out over three chromatographic separations [4] (Table 1). After purification, the preparation was analysed by SDS/PAGE and appeared to contain only the α - and β -subunits (Figure 1).

E.s.m.s. of *P. chrysogenum* AT

AT is coded for by a single gene (*penDE*) which encodes a 40 kDa precursor [6]. This undergoes post-translational cleavage yielding the β - (28 kDa) and α - (11 kDa) subunits. Tobin et al. [6] and Aplin et al. [6a] have proposed a different cleavage site (Gly¹⁰²-Cys¹⁰³) from those proposed by Barredo et al. [5] (Asp¹⁰¹-Gly¹⁰² or Cys¹⁰³-Thr¹⁰⁴).

A typical electrospray mass spectrum of the purified AT (Figure 2) shows a mixture of four species, each generating a

Figure 1 SDS/PAGE (16.5% gel) of purified AT (about 8 μ g)

The molecular masses of the subunits are 28.5 (β -) and 11.5 (α -) kDa. Positions of molecular-mass standards (kDa) are shown on the left.

series of multiply charged ions ($M + H_n$)ⁿ⁺. The mass-transformed spectrum, which reduces each series of multiply charged species to a single molecular mass (Figure 3), indicates that the four species (A, B, C and D in Figure 3) have the relative molecular masses shown in Table 2.

Of the four species observed by e.s.m.s., the molecular masses of components A and D are consistent with the cleavage of a single peptide bond in the preprotein between Gly-102 and Cys-103, in good agreement with the sequencing data obtained by Whiteman et al. [4], and not between Asp-101 and Gly-102 or between Cys-103 and Thr-104 as proposed elsewhere [5]. The other two species (B and C) have molecular masses close to those of the two identified subunits and could not be resolved from them by SDS/PAGE (Figure 1).

The mass of component B was consistent with the modification of the α -subunit by the addition of a phenoxylacetyl group. This was of interest because sodium phenoxylacetate had been added to the growth medium of the *P. chrysogenum* (as a precursor of penicillin V [2]). Assuming consistency in electrospray analysis of the components A and B, the ratio A/B varied between samples of AT prepared from different fermentations ($\approx$ from 1:1 to 1:2). This modification was further investigated by growing *P. chrysogenum* on medium in which the phenoxylacetate had been replaced with phenylacetate or 4-bromophenylacetate respectively. In each case, when AT was purified from these mycelia, the α -subunit had been apparently modified by addition of the respective arylacetyl group (Table 3). Thus, supplementing the growth medium with phenylacetate or 4-bromophenylacetate salts, followed by isolation of the enzyme, resulted in the molecular mass of component B being consistent with the

Figure 2 Electrospray mass spectrum of AT

Four series of multiply charged ions ($M+H_n$)ⁿ⁺ labelled A–D are shown in a range from 750 to 1350 Da, corresponding to the species identified in Figure 3 and Table 2. The *P. chrysogenum* fermentation from which the AT was isolated was grown on medium supplemented with sodium phenoxyacetate.

Figure 3 The mass-transformed spectrum of AT

Two regions are shown: 11 000–12 000 Da showing species A and B, and 27 900–29 000 Da showing species C and D. The *P. chrysogenum* fermentation from which the AT was isolated was grown on medium supplemented with sodium phenoxyacetate.

Table 2 Molecular-mass analysis of AT by e.s.m.s.

The calculated values are the predicted molecular masses of the subunits if the preprotein is cleaved between the residues shown [5–6a]. σD is the standard deviation for each of the molecular-mass determinations over n values. The *P. chrysogenum* fermentation from which the AT was isolated was grown on medium supplemented with sodium phenoxylacetate. For C, the calculated masses are for the β -subunit from which the C-terminal tetrapeptide has been removed.

Component	Calculated mass (Da)			Observed mass (Da)	σD (Da)	n
	Asp ¹⁰¹ -Gly ¹⁰²	Gly ¹⁰² -Cys ¹⁰³	Cys ¹⁰³ -Thr ¹⁰⁴			
A	11 440.68	11 498.20	11 601.34	11 497.35	1.31	7
B				11 631.06	1.21	6
C	28 061.61	28 004.56	27 901.42	28 003.59	1.27	8
D	28 516.72	28 459.67	28 356.53	28 458.20	1.27	6

Table 3 Molecular-mass analysis of the α -subunit of AT by e.s.m.s.

The calculated values are the predicted molecular masses of the α -subunit with and without the addition of an acyl group derived from the precursor. σD is the standard deviation of the molecular-mass determination over n values. The relative intensities are taken from the mass-transformed spectrum. In the case of 4-bromophenylacetic acid, the unacylated α -subunit was not observed in the mixture and the observed mass difference* was calculated using the expected mass of the unacylated α -subunit (11 498.20 Da). The calculated values when no precursor was fed (entry 4) are for the addition of phenylacetyl (†) and acetyl (‡) groups.

Precursor		Observed mass (Da)	σD (Da)	n	Calculated mass (Da)	Approximate relative intensity (%)	Observed mass difference (Da)	Calculated mass difference (Da)
1	Phenoxyacetic	11 631.06	1.31	7	11 633.34	100	133.71	134.14
		11 497.35	1.21	6	11 498.20	50		
2	Phenylacetic	11 615.37	1.89	6	11 617.34	100	116.57	118.14
		11 498.86	7.33	3	11 498.20	95		
3	4-Bromophenylacetic	11 698.43	0.95	4	11 696.24	100	201.15*	197.04
		Not observed			11 498.20			
4	None	11 613.79	1.01	4	11 617.34†	30	116.68	118.14
		11 538.77	2.36	5	11 541.25‡	30		
		11 497.11	1.66	7	11 498.20	100		

addition of a phenylacetyl or bromophenylacetyl group to component A, the α -subunit. AT was also purified from mycelia which had been grown on medium to which no precursor had been added. In this case, the predominant component was consistent with the unacylated α -subunit (entry 4, Table 3), although two other minor species were identified. Corn steep liquor, which is added to the growth medium, is known to contain metabolic precursors of phenylacetate [15], and the species with a molecular mass of 11 613.79 Da is consistent with phenylacetylation of the α -subunit. The species with a molecular mass of 11 538.77 Da is consistent with acetylation of the α -subunit.

Component C was present in variable amounts (the apparent ratio C/D varied from 2:1 to 1:10), and its molecular mass was consistent with the removal of the C-terminal tetrapeptide (NARL) from the β -subunit (calculated mass 28 004.56 Da). Although the action of a non-specific protease during purification cannot be excluded, Muller et al. [16] have identified the C-terminal ARL tripeptide as a putative signal sequence directing AT to microbodies in penicillin-producing *P. chrysogenum* strains.

Use of e.s.m.s. to probe the mechanism of AT

The range of acyl-CoAs accepted by AT in the presence of 6-APA to give 6-acylpenicillins has been investigated by Luengo et al. [17–19]. Straight-chain-saturated acyl-CoAs from hexanoyl to

decanoyl were all shown to produce bioactive penicillins. Some acyl-CoAs unsaturated at the 3 position (hex-3-enoyl and oct-3-enoyl) were also accepted, in contrast with those unsaturated at the 2 position (hex-2-enoyl and oct-2-enoyl). Substituted arylacetyl-CoAs were shown to be acceptable substrates, but cyclohexylacetyl- and cyclopentylacetyl-CoAs were not. We investigated by e.s.m.s the results of incubating AT with a number of acyl-CoAs.

Thus, for example, AT was incubated with 2 equiv. of hex-3-enoyl-CoA for 3 min. The resulting mixture was examined by e.s.m.s. and a new series of peaks was observed indicating the accumulation of an additional component. The mass-transformed spectrum showed that the new component had a molecular mass of 28 558 Da. This was 98 Da higher than the molecular mass observed for the β -subunit (28 459 Da; Figure 4 and Table 4) and is consistent with the transfer of a single hex-3-enoyl group to the β -subunit. The acylation was apparently specific for this subunit, as no further modification of the α -subunit was observed. Addition of large mole excesses of hex-3-enoyl-CoA, in order to increase the proportion of the enzyme acylated, resulted in poor-quality spectra. In those samples of AT containing a significant proportion of component C (28 005 Da), a further peak consistent with apparent acylation of C was observed. In a control experiment, elastase, a serine protease [11], was also incubated with hex-3-enoyl-CoA and no new component consistent with an acylated species was observed.

Figure 4 A region of the mass-transformed spectrum of AT incubated with hex-3-enoyl-CoA

The masses of each component are: C, 28004.8 ± 4.34 Da; D, 28459.32 ± 2.12 Da; E, 28102.74 ± 8.74 Da; F, 28558.57 ± 8.04 Da. The molecular-mass differences for the apparent acylation of components C and D are: (E - C) = 97.98 Da; (F - D) = 99.25 Da. Calculated molecular mass of hex-3-enoyl (C_6H_9O) = 97.13 Da.

Table 4 Observed mass shift of the β -subunit of AT on incubation with acyl-CoAs

The calculated masses for acylation are with each acyl group. The observed values given are for the mass difference (if observed) between the new component and the observed mass of the β -subunit for each incubation. N.D., None detected.

Acyl-CoA	Incubation time (min)	Expected mass difference (Da)	Observed mass difference (Da)
Pentanoyl-	3	84.13	N.D.
Hexanoyl-	3	98.15	101.83
Hex-3-enoyl-	3	96.13	99.25
Heptanoyl-	3	112.18	109.84
Heptanoyl-	6	112.18	105.81
Heptanoyl-	12	112.18	N.D.
Heptanoyl-	3*	112.18	N.D.
Heptanoyl-	3†	112.18	107.92
Octanoyl-	3	126.21	N.D.
Nonanoyl-	3	140.23	N.D.
Phenylacetyl-	3	118.14	118.75
4-Methoxyphenylacetyl-	3	148.17	N.D.
Phenoxyacetyl-	3	134.14	N.D.

* 6-APA added.

† β -Ala added.

6-APA or β -alanine to the incubation of enzyme with heptanoyl-CoA (Table 4). When 6-APA was added to the incubation, the peak corresponding to the acylated β -subunit was no longer observed, consistent with the formation of heptanoyl-6-APA. In a second experiment, β -alanine was added to the incubation and the peak corresponding to the acylated β -subunit was still observed, suggesting that the acylated β -subunit undergoes nucleophilic attack by 6-APA in a relatively specific manner. The addition of 6-APA did not affect the ratio of components A and B, suggesting that the apparent acylation of the α -subunit is permanent.

Acyl-CoAs were each incubated with the enzyme and the resulting mixtures examined by e.s.m.s. (Table 4). The apparent acylation by hexanoyl-, hex-3-enoyl-, heptanoyl- and phenylacetyl-CoAs of the β -subunit is consistent with the transfer of an acyl group to a nucleophilic residue forming a covalent acylenzyme intermediate. This can either undergo hydrolysis to the corresponding acid [20] or undergo rapid attack by 6-APA to give the acylated penicillin. The apparent acylation with heptanoyl-CoA was observed after 3 and 6 min (Table 4) but not after 12 min, consistent with the formation and subsequent hydrolysis of the acyl-enzyme intermediate.

The range of substrates accepted by AT to produce 6-acylpenicillins is wider than that for which the apparent acylation of the β -subunit of AT was observed by e.s.m.s. For an acylenzyme to accumulate (and be observed by e.s.m.s.), the rate of formation of such an intermediate must exceed the rate of hydrolysis. It is possible that for those acyl-CoA for which the putative acyl enzyme was not observed, either rapid hydrolytic turnover of the acyl-CoA had occurred or the rate of enzyme acylation was relatively slow, both of which would preclude accumulation of an observable intermediate.

AT has been reported [3] to have a much greater activity towards acylation of 6-APA than to hydrolysis of the α -aminoadipoyl group from IPN. The concentration of 6-APA may therefore be rate-limiting *in vivo*, resulting in an accumulation of the acylenzyme intermediate. Attack of this intermediate by a residue of the α -subunit in, or bordering on, the active site may explain the observed *in vivo* acylation of the α -subunit. This process may well affect the relative rates of the deacylation of IPN and reacylation of 6-APA.

In summary, e.s.m.s. has confirmed the position of the post-translational cleavage of AT preprotein and identified the apparent modification by acylation *in vivo* and direct observation *in vitro* of the transfer of an acyl group from a number of acyl-CoAs to the β -subunit. Whether this apparent acylation reflects an event in the *in vivo* transformation of IPN to penicillin G cannot be unequivocally stated at this stage and will require further mechanistic studies. This study clearly demonstrates that e.s.m.s. can reveal post-translational modifications that are not readily identifiable by other rapid analytical techniques.

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If the observed acylenzyme is involved in the reaction mechanism, it would be expected to undergo nucleophilic attack by 6-APA. This was investigated by comparing the effects of adding

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