Volume 262, number 2, 342–344 FEBS 08285 March 1990

Acyl coenzyme A: 6-aminopenicillanic acid acyltransferase from *Penicillium chrysogenum* and *Aspergillus nidulans*

P.A. Whiteman, E.P. Abraham, J.E. Baldwin*, M.D. Fleming*, C.J. Schofield*, J.D. Sutherland* and A.C. Willis+

*The Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, *The Dyson Perrins Laboratory and the Oxford Centre for Molecular Sciences, South Parks Road, Oxford, OX1 3 QY, and +MRC Immunochemistry Unit, Department of Biochemistry, South Parks Road, Oxford, OX1 3 QU, UK*

Received 30 January 1990

A study of the final stages of the biosynthesis of the penicillins in *Penicillium chrysogenum* has revealed two types of enzyme. One hydrolyses phenoxymethyl penicillin to 6-aminopenicillanic acid (6-APA). The other, also obtained from *Aspergillus nidulans*, transfers a phenylacetyl group from phenylacetyl CoA to 6-APA. The acyltransferase, purified to apparent homogeneity, had a molecular mass of 40 kDa. It also catalyses the conversion of isopenicillin N (IPN) to benzylpenicillin (Pen G) and hydrolyses IPN to 6-APA. In the presence of SDS it dissociates, with loss of activity, into fragments of ca 30 and 10.5 kDa, but activity is regained when these fragments recombine in the absence of SDS.

Acyltransferase; Penicillin biosynthesis; Isopenicillin N; Benzylpenicillin; *Penicillium*; *Aspergillus*

1. INTRODUCTION

It is now evident that isopenicillin N (IPN) is an intermediate in the biosynthesis of both cephalosporins with a D-α-aminoadipoyl side chain in *Cephalosporium* and *Streptomyces* spp and of penicillins with non-polar side chains, such as phenylacetyl and phenoxyacetyl in *Penicillium chrysogenum* [1]. The transfer of a phenylacetyl moiety from coenzyme A to 6-aminopenicillanic acid (6-APA) by a crude enzyme derived from *P. chrysogenum* was first described by Brunner et al. [2] and Gatenbeck and Brunsberg [3] in 1968. Subsequently Fawcett et al. [4], using ^{3}H-labelled IPN, showed that this penicillin, as well as 6-APA, was converted to benzylpenicillin (Pen G) under similar conditions (fig.1). Later, Luengo et al. [5] described the partial purification of the acyl CoA:6-APA acyltransferase and reported that it did not catalyse the conversion of IPN to Pen G. However, Alvarez et al. [6] obtained a purified protein which catalysed the formation of Pen G from both 6-APA and IPN, consistent with the findings of Fawcett et al. [4]. They concluded that this enzyme was a monomeric protein of 29 kDa. Barredo et al. [7] described a clone from the *P. chrysogenum* genome of a gene encoding a 40 kDa protein, which they believed to be post-translationally processed to give 28.5 and 11.5 kDa fragments. They concluded that the enzyme activity resided solely in the 28.5 kDa fragment. Prompted by these reports we now disclose our findings on the purification and properties of enzymes from *P. chrysogenum* and *Aspergillus nidulans* which play a role in the exchange of penicillin side chains.

Correspondence address: P.A. Whiteman, The Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, UK

2. EXPERIMENTAL

2.1. *Bioassay of enzyme activities*

2.1.1. Phenylacetyl CoA:6-APA acyltransferase

The protein solution for assay contained 50 mM Tris-HCl, pH 7.8, 8 mM DTT, 1 mM phenylacetyl CoA and 0.2 mM 6-APA. After incubation at 32°C for 20 min, the reaction was terminated by the addition of an equal volume of methanol and assayed for Pen G by the hole-plate assay method using *Staphylococcus aureus* NCTC 6571 [8].

2.1.2. Phenylacetyl CoA:IPN acyltransferase

The assay mixture was as that described above, except that 6-APA was replaced with IPN (usually 1 mM). After 1 h the solution was adjusted to pH 2 and twice extracted with butyl acetate. The Pen G was re-extracted into 0.5 mM K_2HPO_4 and assayed as before.

2.1.3. IPN amidohydrolase

The protein solution for assay contained 20 mM Tris-HCl, pH 7.5, 2 mM DTT and usually 0.5 mM IPN. After incubation at 32°C for 1 h, the reaction was terminated by the addition of acetone to final concentration of 70% and the protein removed by centrifugation. The acetone was removed in vacuo, and the pH lowered to 2 and the reaction mixture incubated at 37°C for 2 h to inactivate IPN. The pH was adjusted to 7.5 and the 6-APA formed was phenylacetylated by addition of phenylacetyl chloride. The resulting Pen G was assayed by the hole-plate method [8].

2.1.4. Phenoxymethyl penicillin (Pen V) amidohydrolase

The procedure was similar to that used for the IPN

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00145793/90/$3.50

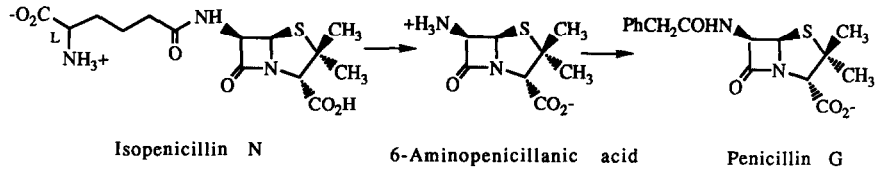

Fig.1. Conversion of penicillin and 6-APA to benzylpenicillin.

amidohydrolase except that Pen V was extracted into butyl acetate (pH 2) before conversion of the 6-APA formed to Pen G.

2.2. *Growth of organisms*

P. chrysogenum (SC 6140, ATCC 2044) was grown and the mycelia were disintegrated essentially as previously described [4,9]. *A. nidulans* (ATCC 28902) was also grown essentially according to previously described procedures [10].

2.3. *Enzyme purification*

Protein purification was carried out at 4°C in the presence of 2 mM DTT. After precipitation of nucleic acids from the crude *Penicillium* extract with protamine sulphate and elution of the enzyme from DEAE-Sephacel, further purification by Affi-gel Blue chromatography yielded two peaks with different enzymic activities (1 and 2). The enzyme in peak 2 was further purified by Phenyl Sepharose chromatography (elution with 40% ethylene glycol), DEAE-5PW (HPLC) or DEAE-Sephacel and by HPLC gel permeation chromatography. 6-APA acyltransferase was purified from *Aspergillus nidulans* by a similar procedure.

2.4. *Amino acid sequencing*

N-terminal amino acid sequences were obtained after purification of the enzyme on SDS-PAGE followed by electroblotting onto Immobilon-P membrane (Millipore UK Ltd). The bands of interest were excised and sequenced directly [11]. Internal sequences were also obtained from this immobilised material by in situ digestion with trypsin followed by reverse phase HPLC [12].

3. RESULTS AND DISCUSSION

The enzyme in peak 1 from the Affi-gel blue column was a Pen V amidohydrolase. Phenylacetyl CoA:6-APA acyltransferase activity, IPN acyltransferase activity, and IPN amidohydrolase activity were restricted to peak 2. The phenylacetyl CoA:6-APA acyltransferase activity was always observed at a much higher level than the IPN amidohydrolase or phenylacetyl CoA:IPN acyltransferase activities, but throughout the purification no separation of the 3 activities was obtained.

Our procedure yielded a protein product with a specific activity for the phenylacetyl CoA:6-APA acyltransferase activity of approx. 100000 pmol/s/mg of protein. A similar procedure applied to the enzyme from *Aspergillus* gave a value of 55000 pmol/s/mg. The corresponding values, for the *Penicillium* enzyme, described by Alonso et al. [13] and Alvarez et al. [6], were 4354 and 310 pmol/s/mg. Our specific activities for the IPN amidohydrolase and phenylacetyl CoA:IPN acyltransferase activities after the DEAE-5PW column were 27 and 414 pmol/s/mg, respectively; but, these values are lower than those that would correspond to V_{max}.

HPLC gel permeation chromatography of the purified *Penicillium* acyltransferase, in the presence of 30% ethylene glycol, gave a molecular mass of 40 kDa. However, on SDS-PAGE two bands were observed corresponding to proteins with molecular mass of ~30 kDa and 10.5 kDa. With the corresponding enzyme from *Aspergillus*, two similar bands were also observed (28 and 10.5 kDa). In the absence of ethylene glycol, gel permeation chromatography of the enzyme gave an apparent molecular mass of ~30 kDa, perhaps explaining the earlier report of Alvarez et al. [6] that a single 29 kDa protein was the active entity.

The N-terminal amino acid sequences of the 30 and 28 kDa proteins were obtained from the *Penicillium* and *Aspergillus* enzymes respectively, together with a sequence of the 10.5 kDa protein from the *Penicillium* enzyme (fig.2a). Some internal sequence data were also obtained for the *Penicillium* enzyme (fig.2b). A high degree of homology between the N-terminal sequences of the larger fragments from the two organisms was observed. These amino acid sequences from the *Penicillium* proteins have enabled probes to be synthesised which have led to the identification of a cDNA clone encoding a 40 kDa protein, which contains the N-terminal sequences of both the 30 and 10.5 kDa proteins, suggesting expression of a single 40 kDa protein which is post-translationally processed [14].

To determine the catalytic role of the 30 and 10.5 kDa fragments we studied the effect of SDS on the 6-APA acyltransferase activity. After 5 min in the presence of 0.01% SDS the enzyme had lost >95% of its activity, but 80% of the lost activity was regained when the concentration of the SDS was reduced to 0.0002% before assay. Further, when a sample which had been incubated in the presence of 0.01–0.1% SDS, was analysed on a HPLC gel permeation column (Waters Protein Pak 300sw) in buffer containing no

P chrysogenum (30kDa) (W) TTAYCQLPNGALQGQNWDFFS(P/A)TKENLI
A nidulans (28kDa) C TTVYCKTPNGALQGQNWDF (a)

P chrysogenum (10.5kDa) MLHILCQGTPFEIGYEHGSA

P chrysogenum (30kDa) ELDPLPDS
ALESTSPSQAYDR
NIIYDHAR (b)

Fig.2. (a) N-terminal amino acid sequences of acyltransferase from *P. chrysogenum* and *A. nidulans*. (b) Internal amino acid sequences of acyltransferase from *P. chrysogenum*.

SDS, ~30% of the original acyltransferase activity was eluted as a single peak.

In contrast, no activity was recovered when the eluting buffer contained 0.05–0.1% SDS. Under these conditions the inactive protein was largely (but not completely) resolved into two proteins of 30 kDa and 10.5 kDa. The results of these experiments indicated that partial denaturation of the enzyme by low concentrations of SDS was concomitant with a dissociation into 30 kDa and 10.5 kDa components and that this process and the resulting loss of activity were reversible.

When solutions containing mainly either the 10.5 kDa or 30 kDa protein were assayed separately, after removal of SDS, a low level of activity was observed. This activity could be attributed to an incomplete separation of the two fragments on the gel permeation column. However, a substantial increase in activity (up to 15-fold) occurred when the two solutions were mixed before assay. It thus appears that reassociation of the 30 kDa and 10.5 kDa proteins is required for acylcoenzyme A:6-APA acyltransferase activity. Thus our results are consistent with a heterodimeric complex being responsible for the acyltransferase activity of *P. chrysogenum*.

Acknowledgements: We thank Eli Lilly, and the SERC for financial support. M.D.F. also thanks the British Marshall Aid Commemoration Commission.

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