ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1987, p. 1675–1682
0066-4804/87/111675-08$02.00/0

Vol. 31, No. 11

Purification to Homogeneity and Characterization of Acyl Coenzyme A:6-Aminopenicillanic Acid Acyltransferase of *Penicillium chrysogenum*

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Received 19 February 1987/Accepted 31 July 1987

The acyl coenzyme A (CoA):6-aminopenicillanic acid (6-APA) acyltransferase of *Penicillium chrysogenum* AS-P-78 was purified to homogeneity, as concluded by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing. The enzyme is a monomer with a molecular weight of 30,000 ± 1,000 and a pI of about 5.5. The optimal pH and temperature were 8.0 and 25°C, respectively. This enzyme converts 6-APA into penicillin by using phenylacetyl CoA or phenoxyacetyl CoA as acyl donors. The pure enzyme showed a high specificity and affinity for 6-APA and did not accept benzylpenicillin, 7-aminocephalosporanic acid, cephalosporin C, or isocephalosporin C as substrates. The enzyme converted isopenicillin N into penicillin G, although with a lower efficiency than when 6-APA was used as the substrate. It did not show penicillin G acylase activity. The acyl CoA:6-APA acyltransferase required dithiothreitol or other thiol-containing compounds, and it was protected by thiol-containing reagents against thermal inactivation. The acyltransferase was inhibited by several divalent and trivalent cations and by *p*-chloromercuribenzoate and *N*-ethylmaleimide. The activity was absent in four different mutants that were blocked in penicillin biosynthesis.

The β-lactam–thiazolidine nucleus of penicillins is formed by cyclization of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to form isopenicillin N (IPN), an intermediate in the biosynthetic pathway that has an L-α-aminoadipyl side chain (Fig. 1) (24). In *Penicillium chrysogenum*, the zwitterionic α-aminoadipyl side chain of IPN can be exchanged for aromatic side chains such as phenylacetyl and phenoxyacetyl groups and can be also removed to yield 6-aminopenicillanic acid (6-APA) (3). 6-APA is the major penicillin derivative that accumulates in precursor-free fermentations (13).

The role of 6-APA in the de novo biosynthesis of penicillin is unclear (23). Demain (8) proposed that the terminal reaction of benzylpenicillin (also known as penicillin G) synthesis, is most likely an exchange of the L-α-aminoadipic acid side chain of IPN for phenylacetic acid, which is activated in the form of phenylacetyl coenzyme A (CoA) (5). However, a two-step reaction involving 6-APA as an intermediate is also possible (Fig. 1). Interconversions between penicillin G and 6-APA or between two hydrophobic penicillins by mycelial suspensions of *P. chrysogenum* have been described (9, 21). Reports on the presence of an acylase in *P. chrysogenum*, which removes the side chain of benzylpenicillin to yield 6-APA, and of an acyltransferase, which catalyzes a direct exchange of side chains between IPN and solvent-soluble penicillins (or between such penicillins and 6-APA), are contradictory (6, 12, 30). It was initially suggested that the penicillin G acylase and the acyl CoA:6-APA acyltransferase activities were associated with a single enzyme (30). However, the acyl CoA:6-APA acyltransferase preparation described by Gatenbeck and Brunsberg (12) did not hydrolyze penicillin G to 6-APA. Moreover, the acylase was apparently unable to produce a detectable hydrolysis of IPN or penicillin N to 6-APA (7, 32). Later, it was shown that an enzyme(s) in crude extracts of *P. chrysogenum* catalyzes the formation of benzylpenicillin from IPN, as well as that from 6-APA, in the presence of phenylacetyl CoA (10).

Attempts to solve the dilemma by isolating the enzyme(s) were hampered by the extreme instability of the enzyme in crude extracts (1). To understand the molecular basis of the regulatory mechanisms that control penicillin biosynthesis (18, 20), it was of utmost interest to purify and characterize the acyltransferase enzyme. In this report we describe the purification to electrophoretic homogeneity and characterization of the acyl CoA:6-APA acyltransferase of *P. chrysogenum* that transforms 6-APA into penicillin G, and the inability of the pure form of this enzyme to hydrolyze penicillin G or to exchange the phenylacetyl side chain of penicillin G with labeled phenylacetyl CoA.

MATERIALS AND METHODS

Microorganisms. *P. chrysogenum* AS-P-78, a strain producing high levels of penicillin, was a gift from Antibióticos, S.A. (León, Spain). *P. chrysogenum* Wis-54-1255, a strain producing low levels of penicillin, was obtained from A. L. Demain (Massachusetts Institute of Technology, Cambridge). Mutants *npe*1 to *npe*8, which are blocked in penicillin biosynthesis, were obtained by mutation of spores of *P. chrysogenum* Wis-54-1255 with nitrosoguanidine (J. M. Cantoral, and J. F. Martín, unpublished data). *Micrococcus luteus* ATCC 9341 and *Escherichia coli* ESS 22-31 (a β-lactam-supersusceptible strain) were used for the routine microbiological determination of penicillin G, as described before (15, 26). All strains were kept lyophilized, and slants were grown in PM1 sporulation medium as described before (15).

Media and culture conditions. Complex seed medium (17) (50 ml in 250-ml flasks) was inoculated with 10^8 conidia of *P. chrysogenum* AS-P-78 and incubated at 25°C for 36 h in an orbital incubator (250 rpm; model G10; New Brunswick Scientific Co., New Brunswick, N.J.); 2.5 ml of this seed

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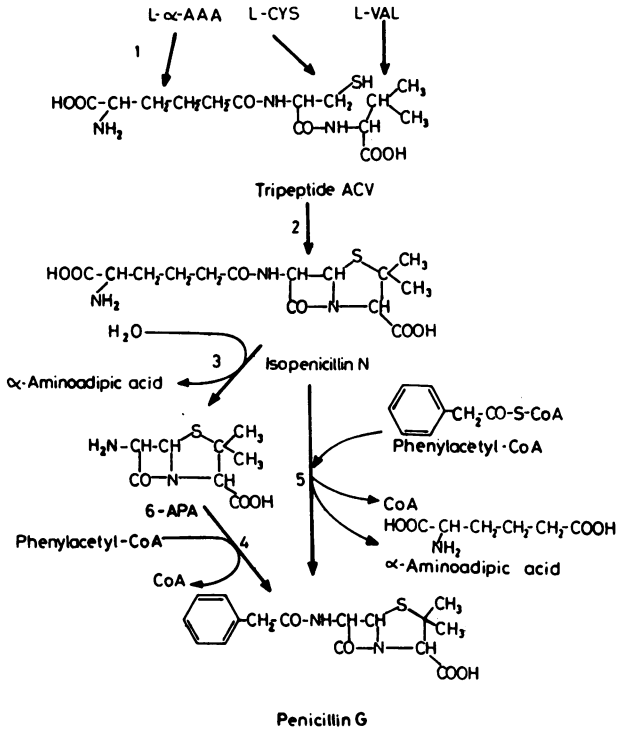

FIG. 1. Biosynthetic pathway of penicillin G. 1, ACV synthetase; 2, IPN synthase (cyclase); 3, IPN amidolyase (6-APA forming); 4, acyl CoA:6-APA acyltransferase; 5, acyl CoA:IPN acyltransferase (transacylase).

culture was added to 50 ml of complex production (CP) medium (17) in 500-ml baffled flasks. The cultures were incubated (250 rpm) at 25°C for 96 to 120 h. Penicillin G was determined in culture broths after centrifugation at 15,000 × *g* for 10 min. Microbiological assays and colorimetric determinations of penicillin G were carried out as described before (17).

6-APA acyltransferase assay. The reaction mixture contained 0.2 mM phenylacetyl CoA, 0.2 mM 6-APA, 5 mM dithiothreitol (DTT), 0.1 M Tris hydrochloride (pH 8.0), and enzyme extract in a final volume of 200 μl. The reaction was incubated at 25°C for 10 min and stopped by the addition of 200 μl of methanol to precipitate the protein. The samples were centrifuged at 5,000 × *g*, and the penicillin G that was formed was assayed in the supernatant. Concentrations of penicillin G as low as 10 ng/ml were detected under our conditions by using *M. luteus* as the test organism. The reaction was linear for at least 10 min. The enzyme activity is given as picokatals (picomoles of penicillin G formed per second) per milligram of protein.

IPN acyltransferase assay. The reaction mixture to measure acyl CoA:IPN acyltransferase was identical to that for measuring 6-APA acyltransferase, but 0.2 mM IPN instead of 6-APA was used. The reaction was carried out for 20 min at 25°C and stopped by the addition of 200 μl of methanol. The penicillin G that was formed was determined by bioassay by using *M. luteus* as the test organism.

Exchange reaction between [^{14}C]phenylacetyl CoA and penicillin G. The reaction mixture contained the following, in a final volume of 200 μl: 0.2 mM [^{14}C]phenylacetyl CoA, 10 mM penicillin G, 5 mM DTT, and 0.1 mg of purified acyltransferase in 0.1 M Tris hydrochloride (pH 8.0). [^{14}C]phenylacetyl CoA was prepared as described by Stadtman (31) by using [1-^{14}C]phenylacetic acid (103 μCi/mmol). The reaction mixture was incubated for 30 min at 25°C and stopped by the addition of 200 μl of methanol. Penicillin G was separated by high-pressure liquid chromatography (16), and the radioactivity that was incorporated into penicillin was determined in a scintillation counter (Phillips; PW4700).

Esterase activity. Nonspecific esterase activity was measured by using *p*-nitrophenylacetate as the substrate. The reaction mixture contained 0.1 mM *p*-nitrophenylacetate, purified enzyme (0.1 mg of protein), and 0.1 M Tris buffer (pH 8.0) in a volume of 1 ml. The reaction was carried out at 25°C for 20 min in a spectrophotometer (410 nm) in parallel with a control reaction without enzyme.

Preparation of cell extracts and purification of the acyltransferase. Cell extracts were prepared from cultures that were grown for 48 h in CP medium, because at this time the enzyme was already formed, but not too much penicillin had accumulated (26). The mycelium was collected by filtration and washed 4 times with a cold (4°C) sodium chloride solution (9 g/liter) to remove the residual penicillin G that adhered to the mycelium. Mycelium (200 g [wet weight]) was suspended in 700 ml of 0.05 M Tris hydrochloride buffer (pH 8.0) containing 5 mM DTT (TD buffer). The mycelium was disrupted either by sonication or mechanical disintegration. Sonication was carried out in a sonifier (B-12; Branson Sonic Power Co., Danbury, Conn.) for periods of 20 s with intervals of 1 min; the sample was kept in an ice bath. Mechanical disintegration was carried out in a disintegrator (Braun, Melsungen, Federal Republic of Germany) by using Ballotini glass beads (type V; diameter, 450 to 500 μm; Sigma Chemical Co., St. Louis, Mo.) for periods of 30 s at intervals of 15 s with refrigeration by liquid CO_2. The extract was centrifuged at 20,000 × *g* for 30 min at 4°C, and the traces of penicillin G remaining in it were removed by ammonium sulfate precipitation (see below) or by filtration of the crude extract through a coarse Sephadex G-25 column (10 by 1 cm) equilibrated with TD buffer.

All the following purification steps were carried out at 4°C, and the enzyme was always kept in the presence of 5 mM DTT (TD buffer) to protect the activity.

Protamine sulfate and ammonium sulfate precipitation. Nucleic acids and nucleoproteins were removed from the extract by slowly adding 27 ml of a 10% (wt/vol) solution of protamine sulfate in 0.05 M Tris hydrochloride (pH 8.0) to 640 ml of cell extract (final concentration of protamine sulfate, 0.4% [wt/vol]). After stirring for 45 min at 4°C, the precipitate was removed by centrifugation at 20,000 × *g*. The supernatant was fractionated by precipitation with ammonium sulfate (enzyme grade). The pH of the solution was adjusted to 8.0 after each addition of ammonium sulfate. The proteins that precipitated in the fractions of 0 to 40% and 40 to 55% ammonium sulfate saturation were collected by centrifugation at 20,000 × *g* for 30 min at 4°C.

Hydrophobic interaction chromatography on phenyl Sepharose CL-4B. The protein in the 40 to 55% ammonium sulfate fraction was dissolved in TD buffer supplemented with 1 M ammonium sulfate and applied to a phenyl Sepharose CL-4B column (1.8 by 16 cm) equilibrated with the same buffer. The unbound proteins were removed by washing with TD buffer (flow rate, 5 ml/min) until no more protein was released. The acyltransferase activity was retained in the column and eluted with 40% ethylene glycol in 0.05 M Tris hydrochloride (pH 8.0).

Ion-exchange chromatography on DEAE-Sephacel. The active fractions from the previous step were pooled and applied to a DEAE-Sephacel column (1.5 by 20 cm) equili-

TABLE 1. Purification of the acyl CoA:6-APA acyltransferase of *P. chrysogenum* AS-P-78

Purification step	Acyltransferase activity (pkat)	Protein (mg)	Sp act (pkat/mg of protein) with the following substrate:		Recovery (%)	Purification (fold)
			6-APA	IPN		
Crude extract	3,888	3,240	1.2	0.2	100	1.0
Protamine sulfate supernatant	3,630	1,160	3.1	0.6	93	2.6
Ammonium sulfate (40 to 55%) precipitate	2,510	410	6.1	1.1	64	5.1
Phenyl Sepharose	1,060	14	75.7	16.3	27	63.1
DEAE-Sephacel	740	3.5	211.4	38.2	19	167.0
Sephadex G-75	310	1	310	55.3	8	258.3

brated with TD buffer, and the acyltransferase activity eluted with a linear gradient of NaCl (0 to 0.25 M) in TD buffer (flow rate, 0.25 ml/min).

Gel filtration through Sephadex G-75. The active fractions from the DEAE-Sephacel column were precipitated with ammonium sulfate (70% saturation), and the pellet was dissolved in TD buffer (3 ml) and applied to a coarse Sephadex G-75 column (2.6 by 70 cm) that was previously equilibrated with the same buffer. The acyltransferase was eluted with TD buffer (flow rate, 9 ml/h).

Identification of the reaction product. The reaction product was identified as penicillin G by (i) microbial susceptibility tests, (ii) degradation with penicillinase, and (iii) thin-layer chromatography and high-pressure liquid chromatography after the reaction product was extracted with solvent. Microbial susceptibility tests against *M. luteus* ATCC 9341 *E. coli* ESS 22-31, *Bacillus subtilis* ATCC 6633, and *Klebsiella pneumoniae* ATCC 29665 were carried out as described before (16, 26, 27). The antibiotic that was formed showed activity against *B. subtilis*, *M. luteus*, and *E. coli*, but not against the penicillin-resistant strain of *K. pneumoniae*. A sample of the reaction product was treated with penicillinase (*Bacillus cereus* UL1 penase; 10 g/100 μl of reaction sample). The penicillinase destroyed the antibiotic that was formed. Further characterization of the reaction product by thin-layer chromatography and high-pressure liquid chromatography was carried out as described before (16). In the thin-layer chromatographic procedure, the reaction product comigrated with pure penicillin G. In the high-pressure liquid chromatographic procedure the antibiotic activity eluted in a peak with a retention time of 12.3 min, which is identical to that of authentic penicillin G.

Electrophoresis and in situ determination of acyltransferase. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was carried out as described by Laemmli (14) by using 11% polyacrylamide gels. Nondenaturing gels for in situ determination of the acyltransferase were made without SDS. After electrophoresis, the gel was cut into 1.0-cm-wide slabs, and each slab was divided into fragments of 0.5 cm. Each fragment was chopped up in 1 ml of 50 mM Tris hydrochloride buffer (pH 8.0) containing 5 mM DTT, 0.2 mM phenylacetyl CoA, and 0.2 mM 6-APA. After incubation for 60 min at 25°C, the penicillin G that was formed was detected by assay against *E. coli* ESS 22-31 or *M. luteus*, as indicated above.

Isoelectric focusing. Isoelectric focusing was carried out in 1 mm polyacrylamide gels bound to glass plates (230 by 115 mm) with a flat-bed apparatus (FBE 3000; Pharmacia Fine Chemicals, Piscataway, N.J.) and a constant power source (Shandon Vokam 2000-300-150; LKB Instruments, Inc., Rockville, Md.), according to the instructions of the manufacturer. Samples were loaded onto the gels and electrofocused for 60 min at 1,600 V. Proteins with known pIs in the range of 3.5 to 9.3 (broad pI calibration kit; Pharmacia) were used as controls.

Molecular weight determination. The molecular weight of the active enzyme was estimated by gel filtration in a column of Sephadex G-100 (2.6 by 70 cm). The column was calibrated with a set of proteins with known molecular weights: cytochrome *c* (M_r, 12,500), carbonic anhydrase (M_r, 29,000), ovalbumin (M_r, 45,000), and bovine albumin (M_r, 66,000). The proteins were eluted with TD buffer (flow rate, 15 ml/h). The molecular weight of the denatured protein was measured by SDS-PAGE (11% polyacrylamide), with trypsinogen (M_r, 24,000), carbonic anhydrase (M_r, 29,000), glyceraldehyde-3-phosphate dehydrogenase (M_r, 36,000), ovalbumin (M_r, 45,000), and bovine albumin (M_r, 66,000) used as standards.

Chemicals. Penicillin G (sodium salt) was provided by Antibióticos, S.A., León, Spain; IPN and 6-APA were gifts from Gist-Brocades, Delft, The Netherlands. Penicillin N was provided by H. H. Peter (CIBA-GEIGY, Basel, Switzerland). Phenylacetic acid was obtained from Aldrich Chemical Co. (Steinheim, Federal Republic of Germany). [1-^{14}C]phenylacetic acid (103 μCi/mmol) was donated by B. Meesschaert (Ostend, Belgium). Phenylacetyl CoA was synthesized by the method described by Stadtman (31). CoA and the proteins used as molecular weight markers were obtained from Sigma. Sephadex G-75 and G-100, Phenyl-Sepharose CL-4B, and DEAE-cellulose were acquired from Pharmacia. All other reagents were of analytical quality.

RESULTS

Purification of the acyltransferase to homogeneity. The specific activity of the enzyme in the crude extract was increased 2.6-fold by the removal of nucleic acids and nucleoproteins with protamine sulfate. Most acyltransferase activity was recovered in the 40 to 55% ammonium sulfate saturation fraction (Table 1).

A very good purification was obtained by the hydrophobic interaction chromatography on phenyl Sepharose CL-4B. The overall recovery of activity was reduced to 27% after this step, but the accumulated purification increased up to 63-fold. After ion-exchange chromatography through DEAE-Sephacel, the 6-APA acyltransferase reached a 176-fold purification with a good recovery. The final purification (258-fold) was achieved by gel filtration through Sephadex G-75. A symmetrical peak of protein was obtained in the late part of the eluted fractions that corresponded to active 6-APA acyltransferase.

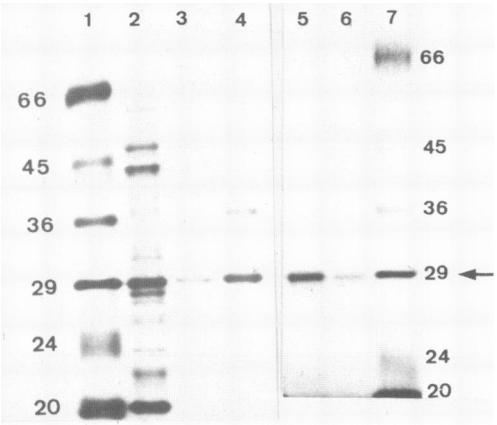

FIG. 2. SDS-PAGE of fractions from different steps of purification. Lanes 1 and 7, molecular weight standards (in thousands); lane 2, eluate from the phenyl Sepharose CL-4B column; lanes 3 and 4, fraction numbers 32 and 36 from the DEAE-Sephacel column; lanes 5 and 6, two different amounts (approximately 16 and 8 μg, respectively) of the pure acyltransferase after Sephadex G-75 gel filtration. The acyltransferase band is indicated by an arrow.

PAGE and isoelectric focusing. Samples from all the purification steps were analyzed by SDS-PAGE. A band that migrated with the same mobility as carbonic anhydrase was enriched throughout the purification procedure (M_r, 29,000) (Fig. 2). The acyltransferase band was observed after hydrophobic interaction chromatography on phenyl Sepharose CL-4B and became the major band after DEAE ion-exchange chromatography (Fig. 2). A single protein band was obtained after Sephadex G-75 gel filtration. This protein band corresponded to the acyltransferase, as concluded from in situ enzyme assays after nondenaturing PAGE, as indicated above.

The acyltransferase gave a single band after isoelectric focusing. The isoelectric point of the enzyme was about 5.5.

Molecular weight. The active enzyme purified to electrophoretic homogeneity showed a K_{av} value of 0.45 by gel filtration on Sephadex G-100 that corresponded to a relative molecular mass of 30,000 ± 1,000 (Fig. 3). This value agrees with the relative mass of the SDS-denatured protein calculated by SDS-PAGE (M_r, 29,000), which suggests that the enzyme is a monomer polypeptide.

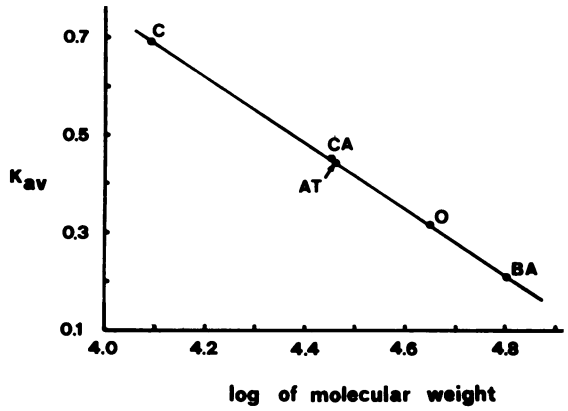

FIG. 3. Determination of the molecular weight of the 6-APA acyltransferase by Sephadex G-100 gel filtration. Abbreviations: C, Cytochrome *c*; CA, carbonic anhydrase; AT, acyltransferase (arrow); O, ovalbumin; BA, bovine albumin.

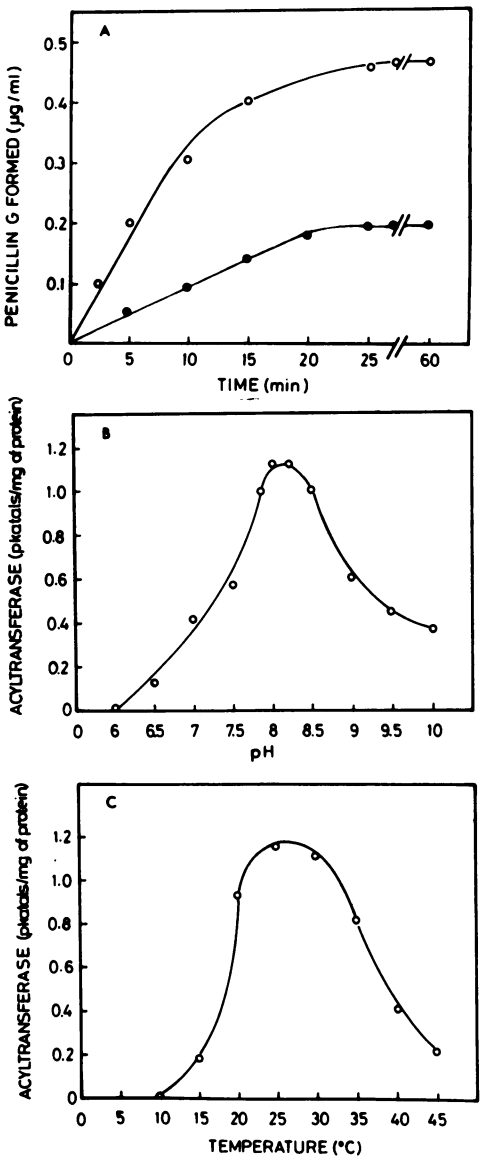

FIG. 4. Time course of the acyltransferase reaction by using 6-APA (○) and IPN (●) as substrates (A), optimal pH (B), and temperature (C) of the acyltransferase. Phosphate buffer (100 mM) was used in the range between pH 6.0 and 7.5, and Tris hydrochloride (100 mM) was used between pH 7.8 and 10.

Optimal pH and temperature. Under the conditions described above, the reaction was linear for 10 min (Fig. 4A). The activity of the enzyme was tested at pH values between 6.0 and 10.0 by using 100 mM phosphate buffer in the pH range of between 6.0 and 7.5 and 100 mM Tris hydrochloride between pHs of 7.8 and 10. The optimal enzyme activity was observed at pH 8.0 and 8.2. Below pH 7.0 or above pH 9.5 the activity was very low (Fig. 4B).

The optimal temperature was 25°C, although the enzyme retained good activity up to 30°C (Fig. 4C).

Requirement of cofactors. Acyl CoA:6-APA acyltransferase required DTT for activity. A concentration-dependent increase in activity was observed with up to 3 mM DTT (Fig. 5). The requirement was not specific for DTT. Some thiol-containing molecules such as dithioerythritol, CoA, and dimercaptopropanol were used as cofactors instead of DTT,

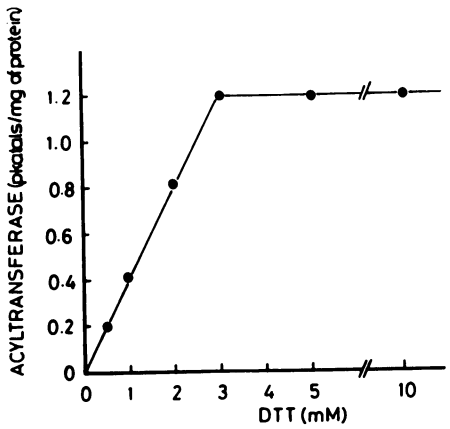

FIG. 5. Effect of DTT on acyltransferase activity.

with 100% activity compared with that of DTT (Table 2). Other thiol-containing compounds such as β-mercaptoethanol and reduced glutathione were used very poorly as cofactors. The enzyme did not require Fe^{2+}, ascorbic acid (cofactors of the IPN synthase of this same organism), or ATP. It did not appear to require α-ketoglutarate, which is a well-known cofactor of the deacetoxycephalosporin C synthase of *Acremonium chrysogenum*.

Stability of the enzyme: protection by DTT. Both crude extracts and the pure 6-APA acyltransferase were kept frozen at −20°C for several months without loss of activity. At 4°C the enzyme lost 50% of its activity in 30 h (Fig. 6A). When the enzyme was kept at 4°C before the assay in the presence of DTT (5 mM) for at least 3 h, the enzyme gained activity and maintained a higher activity than did control preparations without DTT, although there was a loss of activity with time. This protection effect appears to be different from the cofactor requirement that is observed when a thiol-containing compound is added during the reaction.

6-APA acyltransferase in vitro was very sensitive to high temperatures. It lost 50% of the activity when incubated for 2 h at 25°C, and was completely inactivated when it was kept at 35°C for 2 h (Fig. 6B). DTT (5 mM) partially protected the acyltransferase against thermal inactivation. In the presence of DTT, about 85% of the activity was retained after incubation for 2 h at 25°C, and 65% remained after incubation for 2 h at 35°C (Fig. 6B).

Substrate specificity. The pure 6-APA acyltransferase showed a high substrate specificity and affinity for the acceptor substrate. The apparent K_m for 6-APA was 9.3 μM, and for IPN the apparent K_m was 21 μM (Table 3). Penicillin N was poorly used as a substrate. Benzylpenicillin, 7-aminocephalosporanic acid, cephalosporin C, and isocephalosporin C (containing an L-α-aminoadipyl side chain) did not function as substrates. The apparent K_m for phenylacetyl CoA was 6 μM. Phenoxyacetyl CoA was used with a similar affinity. Free phenylacetate or phenoxyacetate were not used as substrates. Pure preparations of the enzyme did not hydrolyze *p*-nitrophenylacetate and did not exchange the phenylacetyl side chain of penicillin G with labeled phenylacetyl CoA. Furthermore, the enzyme did not hydrolyze penicillin G or penicillin V to 6-APA and the corresponding side chains; i.e., it did not show penicillin acylase activity.

TABLE 2. Effect of thiol-containing compounds on acyltransferase activity

Thiol compound (5 mM)	Acyltransferase activity (pkat/mg of protein)	Acyltransferase (% of activity)
DTT	80	100
Dithioerythritol	80	100
β-Mercaptoethanol	16	20
Dimercaptopropanol	80	100
CoA	80	100
Glutathione	4.7	6
Thionitrobenzoic acid	0	0

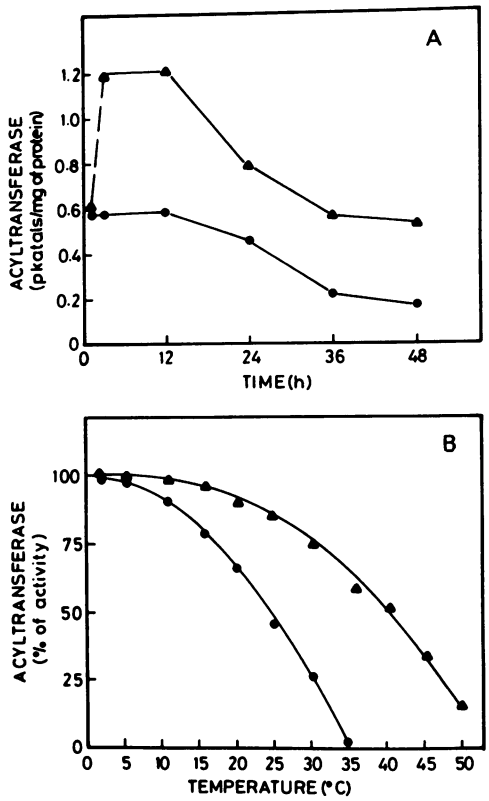

FIG. 6. Protection by DTT of the inactivation of acyltransferase at 4°C with time (A) and at increasing temperatures for 2 h (B). Symbols: ●, control without DTT; ▲, samples kept in the presence of 5 mM DTT. In the control samples, DTT was added only at the time of the reaction, whereas the protected samples were maintained in the presence of DTT at all times, and no supplementary DTT was added during the reaction.

Effect of cations and other inhibitors. The pure acyltransferase did not require cations for maximal activity. Monovalent cations (Na^+, K^+, Li^+, Rb^+, Cs^+; 1 mM) did not affect the enzyme activity. Several divalent and trivalent cations exerted a strong inhibitory effect on enzyme activity. Thus, Zn^{2+}, Pb^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, and Ni^{2+} produced a 100% inhibition at a 1 mM concentration (Table 4). The enzyme was also inhibited to a different extent by several agents that react with thiol groups; e.g., 99% inhibition was produced by 2 mM *p*-chloromercuribenzoate, 87% by 2 mM *N*-ethylmaleimide, and 22% by 2 mM 5,5′-dithiobis(2-nitrobenzoic acid).

Time course of acyltransferase activity during culture. The time course of the acyltransferase activity during the culture of *P. chrysogenum* AS-P-78 is indicated in Fig. 7. The activity of this enzyme in the cells was low at 24 h and increased rapidly from 24 to 48 h. The highest specific

TABLE 3. Kinetic parameters of the acyl CoA:6-APA acyltransferase[a]

Substrate	K_m (μM)	V_{max} (pkat/mg of protein)
Phenylacetyl CoA	6.0	960
6-APA	9.3	740
IPN	23.0	138

[a] Values are averages of three determinations.

activity was observed at 48 h and decreased thereafter, although it still maintained a significant activity at 120 h when penicillin production ceased.

Lack of 6-APA acyltransferase activity in *npe* mutants. Four of the *npe* mutants isolated in our laboratory lacked acyltransferase activity but retained the ability to synthesize the tripeptide α-aminoadipyl-cysteinyl-valine, and showed a normal IPN synthase activity. These results strongly support the involvement of 6-APA acyltransferase activity in penicillin biosynthesis and the requirement of this activity for penicillin production.

DISCUSSION

The acyl CoA:6-APA acyltransferase that converts 6-APA (and IPN) into penicillin G was purified to electrophoretic homogeneity by a combination of chromatographic techniques (Table 1 and Fig. 2). A 258-fold purification of the enzyme was attained up to a specific activity of 310 pkat/mg of protein, despite the known instability of the enzyme (1), by carrying out all the purification steps in DTT-containing TD buffer. The acyltransferase was also homogeneous in isoelectrofocusing (pI, about 5.5). The pure enzyme had an activity of 310 pkat/mg of protein (i.e., 20 nmol/min mg of protein). The IPN synthase of the same organism showed 205 pkat/mg of protein (i.e., 12.3 nmol/min mg of protein) (24). The acyltransferase was found in two different penicillin-producing strains of *P. chrysogenum* (Wis-54-1255 and AS-P-78) but was absent in four *npe* mutants isolated in our laboratory, suggesting that the acyl CoA:6-APA acyltransferase is truly involved in penicillin biosynthesis (19a). The optimal temperature (25°C) was the same temperature at which the production of penicillin was best.

The time course of acyltransferase during the culture is compared (Fig. 7) with the changes in IPN synthase, which

TABLE 4. Effect of cations on acyltransferase activity

Cation added (1 mM)	Acyltransferase activity (pkat/mg of protein)	Acyltransferase (% of inhibition)
None (control)	64	0
Na^+	64	0
K^+	64	0
Li^+	64	0
Cs^+	64	0
Rb^+	64	0
Zn^{2+}	0	100
Mn^{2+}	7.4	89
Pb^{2+}	0	100
Fe^{2+}	0	100
Fe^{3+}	0	100
Ba^{2+}	32	50
Co^{2+}	0	100
Ca^{2+}	50	22
Hg^{2+}	0	100
Ni^{2+}	0	100
Sn^{2+}	1.2	99

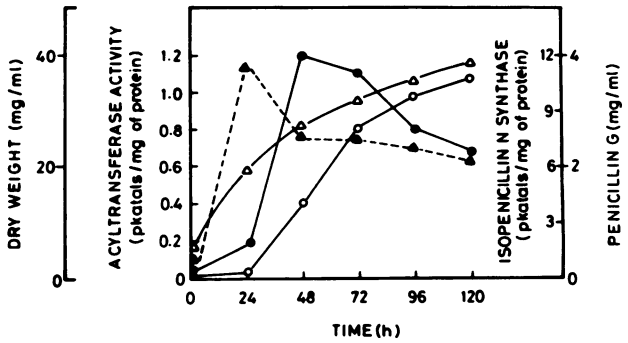

FIG. 7. Time course of acyltransferase activity and penicillin production during the culture of *P. chrysogenum* in CP medium. Symbols: △, dry weight; ○, penicillin G; ●, penicillin acyltransferase; ▲, IPN synthase.

reached a maximal activity at 24 h (24). A sequential formation of both enzymes following growth of *P. chrysogenum* was observed. The large increase in 6-APA acyltransferase between 24 and 48 h of growth was closely associated with the onset of penicillin biosynthesis, whereas IPN synthase formation preceded formation of the antibiotic. The sequential formation of the biosynthetic enzymes of cephalosporins (25) and other secondary metabolites (19) appears to be a general phenomenon in fungi. The specific activity of the acyl CoA:6-APA acyltransferase declined at the same time as the penicillin production rate (Fig. 7).

The acyl CoA:6-APA acyltransferase showed a relative molecular weight of 30,000 ± 1,000 by Sephadex G-100 gel filtration and 29,000 by SDS-PAGE (Fig. 2 and 3). It appears to be a monomer polypeptide since the SDS-denatured form of the enzyme showed the same relative molecular weight as the nondenatured form. Similar conclusions have been obtained with the IPN synthase of *P. chrysogenum* (24) and *A. chrysogenum* (2). The molecular weight of the acyltransferase (30,000) was slightly lower than that of the IPN synthase of *P. chrysogenum* (M_r, 39,000) (24) and *A. chrysogenum* (M_r, 38,416) (28).

The acyltransferase showed a high affinity and specificity for 6-APA (K_m, 9.3 μM). It did not accept 7-aminocephalosporanic acid, benzylpenicillin, cephalosporin C, or isocephalosporin C. The affinity of the enzyme for IPN (Table 3) and penicillin N (data not shown) as substrates was smaller than that for 6-APA.

The presence of a penicillin acylase and an acyltransferase in *P. chrysogenum* was observed several years ago (6, 12); however, the role of these enzymes in penicillin biosynthesis was obscure. It was initially suggested that the penicillin acylase and acyltransferase activities were associated with a single enzyme (30). Gatenbeck and Brunsberg (12) partially purified a soluble acyl CoA:6-APA acyltransferase and observed that it was not able to hydrolyze penicillin G. Similarly, Pruess and Johnson (22) partially purified a penicillin G:6-APA acyltransferase that did not catalyze the hydrolysis of penicillin G. Furthermore, this enzyme preparation could not utilize penicillin N as a substrate. Unfortunately, IPN was not tested by these investigators. Our results indicate that the pure acyl CoA:6-APA acyltransferase lacks penicillin acylase activity. The biosynthetic (6-APA acyltransferase) and degradative (penicillin acylase) activities appear to be in two different enzymes, at least in *P. chrysogenum* AS-P-78. However, the possibility that the numerous industrial mutants of *P. chrysogenum* have dif-

ferent related activities is not excluded. Mutants of *P. chrysogenum* have been described which are able to exchange the D-α-aminoadipic acid side chain of cephalosporin C with phenoxyacetic acid (11).

Our crude and pure preparations of the enzyme were able to directly convert IPN into penicillin G, as reported by Abraham (1). However, the enzyme showed a lower affinity for IPN than for 6-APA. These results are consistent with a two-step conversion of IPN into penicillin G (Fig. 1). An initial step appears to involve the cleavage of IPN to 6-APA followed by the acylation of 6-APA. Both enzymatic steps are carried out by the pure acyltransferase, suggesting that 6-APA may be an enzyme-bound intermediate which is not usually released if the 6-APA acylation can proceed.

The presence of an IPN amidolyase (6-APA forming) has been suggested previously (1, 23), and it would be consistent with the accumulation of α-aminoadipic acid (26) and 6-oxopiperidine-2-carboxylic acid (4) in fermentation broths, but the existence of such an enzyme has not been unequivocally proved. The likelihood of an association between IPN amidolyase (6-APA forming) and acyl CoA:6-APA acyltransferase in an enzyme complex has been suggested (23).

According to our hypothesis, the transacylation of IPN to penicillin G is carried out in two steps by two enzyme activities that are located in a single protein. The loss of one cofactor or the partial denaturation of the 6-APA-forming activity during purification may explain why the enzyme acylates 6-APA more efficiently than it transacylates IPN. The relationship between these two activities is being investigated further.

ACKNOWLEDGMENTS

This study was supported in part by grants from CAICYT, Madrid, and Gist-Brocades NV, Delft, The Netherlands.

We thank B. Koekman for providing IPN and 6-APA, F. Salto for penicillin G, H. Sauber for isocephalosporin C, and H. Peter for penicillin N. We are indebted to M. P. Puertas, B. Martín, and N. Calvo for excellent technical assistance.

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