THE BIOSYNTHESIS OF PENICILLINS: ACYLATION OF 6-AMINOPENICILLANIC ACID

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The final stage in the biosynthesis of penicillins is thought to be the substitution of the L-α-aminoadipyl side chain of isopenicillin N by acyl groups which arise endogenously or which are supplied exogenously as acids or their precursors (Demain, 1966). The mechanism of the acyl substitution is not known but in the absence of exogenously supplied acyl groups 6-aminopenicillanic acid (6-APA) accumulates in the mycelia of penicillin-producing fungi (Batchelor et al., 1959) suggesting that the various penicillins arise by acylation of 6-APA produced from isopenicillin N by hydrolysis rather than by direct transfer with the α-aminoadipyl side chain of isopenicillin N. Wolff and Arnstein (1960), however, failed to observe a clear precursor-product relationship between 6-APA and penicillin G.

An enzyme in cell-free extracts of Penicillium chrysogenum which transfers the acyl side chain between two different penicillins and catalyzes 6-APA exchange with penicillins was reported by Peterson and Wideburg (1960) and Pruess and Johnson (1967). Such an enzyme might be expected to transfer acyl groups from their S-coenzyme A derivatives to 6-APA. This activity could not be demonstrated at the degree of purity obtained by Pruess and Johnson (1967) but purer preparations of penicillin acyltransferase showed strong direct acylating activity. The relevance of this activity to penicillin biosynthesis is discussed.

MATERIALS AND METHODS

^{35}S-benzyl penicillin (530 mc/mmole), ^{35}S-6-aminopenicillanic acid (^{35}S-6-APA) and other ^{35}S-penicillin derivatives were prepared as described by Pruess and Johnson (1965). Acyl thiol esters, including S-phenylacetyl-1-^{14}C CoA (from phenyl acetic acid-1-^{14}C, 22.5 mc/mmole, Nuclear Chicago), were prepared from the acid anhydrides or by the mixed anhydride method (Stadtman, 1957). Phenylacetyl AMP was prepared according to Moldave and Meister (1957) and phenylacetyl phosphate was made by the method used for acetyl phosphate (Kornberg et al., 1956). Where appropriate, compounds were assayed by the hydroxamate method (Lipmann and Tuttle, 1945).

P. chrysogenum 51-20F3 was grown in corn-steep liquor medium for 48-72 hr under conditions described by Pruess and Johnson (1967). Harvested mycelium was washed well with water and pressed between filter papers until superficially dry. The pressed mycelium (10 g) was suspended in 40 ml of 0.05 M potassium phosphate buffer at pH 8.0 containing 5 mM dithioerythritol (DTE) and extruded twice with a French Press at 10,000 psi. After centrifuging at 35,000 X g for 30 min at 2°, the supernatant was adjusted to pH 8.0 and fractionated between 35 and 50% saturation with ammonium sulfate. The precipitated material was dissolved in 2 ml of 0.01 M phosphate buffer, pH 8.0, containing 1 mM dithioerythritol and passed through a 15 x 2.5 cm column of Sephadex G-25 which had been equilibrated with the same buffer. The eluate (6 ml) was absorbed onto a 2.5 x 2.5 cm column of DEAE-cellulose and the column washed with 20 ml of 0.01 M phosphate, pH 8.0, containing 1 mM DTE. The acyltransferase was then eluted in 4.5 ml with the same buffer containing 0.25 M KCl and stored at -70°.

Penicillin acyltransferase activity was assayed by the APA exchange reaction (Pruess and Johnson, 1967). The enzyme was incubated at 37° with ^{35}S-benzyl pen-

icillin plus 2 mM unlabeled benzyl penicillin, 7.5 mM 6-APA, 2 mM dithioerythritol, 2 mM EDTA and 0.05 M potassium phosphate buffer, pH 8.0, in a total volume of 30 µl. At the appropriate time a 10 µl sample was added to 5 µl of 0.4 M citrate buffer, pH 5.0, to stop enzyme activity. Portions (5 µl) of the mixture were chromatographed on Whatman No. 1 paper which had been impregnated with phosphate buffer, pH 6.0, as described by Pruess and Johnson (1967) and run in an ethanol/n-butanol/28% ammonium sulfate solution, 2/1/1 system (cf. Tardrew and Johnson (1959). In this system, benzyl penicillin, penicilloic acid and 6-aminopenicillanic acids had R_f values of 0.75, 0.46 and 0.58, respectively. After radioautography, the portion of the paper corresponding to ^{35}S-6-aminopenicillanic acid was identified and its radioactivity measured by liquid scintillation counting with reference to standards of ^{35}S-benzyl penicillin. Direct acylation of 6-APA was assayed by incubation of the enzyme at 37° with ^{35}S-6-APA, plus unlabeled 6-APA, acyl-CoA or other acyl-donor, DTE and EDTA in 0.05 M potassium phosphate buffer at pH 7.5. Concentrations of reactants are stated in the text. Samples were removed, chromatographed and the radioactivity corresponding to ^{35}S-penicillins counted as described above.

RESULTS AND DISCUSSION

Although crude extracts of mycelium showed considerable APA exchange activity, acylation of 6-APA by S-phenylacetyl CoA could not be demonstrated with certainty. Since part of the difficulty may have been due to thiol ester hydrolysis the penicillin acylase was purified and re-examined. The eluate from the DEAE column contained 2.2 mg of protein/ml and produced 42 mµmoles of ^{35}S-6-APA/min/mg of protein in the transfer reaction between ^{35}S-penicillin G and unlabeled 6-APA. This represented a 20-fold increase in activity over that of the original supernatant with retention of 55% of the activity. The preparation also catalyzed

Table I

Specificity of Acylating Enzyme

Incubation mixtures contained 5 mM DTE, 2 mM EDTA, 0.05 M potassium phosphate buffer and 22 μg of enzyme protein at pH 7.4 in a volume of 30 μl. Additions were: for specificity of acyl activation, 1 mM ^{35}S-6-APA (150,000 cpm per incubation) and 5 mM acyl compound; for acyl group specificity, 1 mM ^{35}S-6-APA (150,000 cpm per incubation) and 5 mM S-acyl CoA; for specificity of acyl acceptor, 5 mM S-phenylacetyl-1-^{14}C CoA (120,000 cpm per incubation) and 1 mM acceptor. Control determinations omitted enzyme, acyl donor or acyl acceptor. Incubation mixtures were analyzed as described in the experimental section. All penicillins had the same R_f (0.75) as benzyl penicillin. The R_f values of the ^{14}C spots formed from acyl acceptors other than 6-APA are shown.

ACYL ACTIVATION Acyl donor	mμmoles benzyl penicillin formed/30 min/30 μl
S-phenylacetyl CoA	12.3
phenylacetic acid	0
phenylacetyl phosphate	0
phenylacetyl adenylate	0
phenylacetyl glycine	0
ACYL GROUP S-CoA ester of	
phenyl acetic acid	11.7
β,γ-hexenoic	2.8
caproic	0.69
valeric	0.53
acetoacetic	0.74
acetic	0
palmitic	0
benzoic	0
isobutyric	0.19
DL-α-methoxyphenylacetic	0.25

ACYL ACCEPTOR Acceptor	R_f of ^{14}C spot	mμmoles ^{14}C compound formed/30 min/30 μl
6-APA	0.75	11.4
D-4-carboxy-5,5-dimethyl-α-amino-2-amino-2-methylthiazolidine (CDAT)	0.80	2.3
penicic acid amide	0.79	7.0
penicic acid hydroxamate	0.86	11.4
penicic acid		0
7-aminocephalosporanic acid		0
7-aminodeacetyl-cephalosporanic acid		0
L-glycine		0
L-glutamate		0
L-glycyl glycine		0
L-glycyl amide		0
L-alanyl amide		0
L-valine methyl ester		0

the direct acylation of ^{35}S-6-APA by S-phenylacetyl CoA to give ^{35}S-benzyl penicillin which was characterized by its chromatographic identity with authentic benzyl penicillin and the formation of ^{35}S-benzyl penicilloic acid and ^{35}S-benzyl penilloic acid following successive treatments with alkali and acid (Pruess and Johnson, 1965). The optimum pH for the acylation was 7.4 in 0.05 M potassium phosphate buffer. Activity was negligible at pH 6.0 and below this pH the enzyme was rapidly destroyed. In the presence of 2 mM EDTA and 5 mM DTE and with an incubation period of 15 min, maximum acylating activity was given with 1 mM 6-APA as acceptor and 4 mM S-phenylacetyl CoA as donor with a K_m for 6-APA of 2×10^{-4} M. Under the optimum conditions the extract formed 38 mμmoles of benzyl penicillin/min/mg protein. Both the penicillin acyltransferase and direct acylating activities of the P. chrysogenum extract showed a requirement for -SH. In the absence of EDTA, 20 mM DTE was required for the maximum level of both activities. Addition of 2 mM EDTA reduced the concentration of DTE required for maximum activity to 1.5 mM.

The acyl donor specificity of the enzyme appears to be restricted to the activation of the phenylacetyl group as the thiol ester, since the acid itself and its phosphate, adenylate and glycine conjugate failed to act as substrates for the enzyme (Table I). S-phenylacetyl CoA was by far the best acyl donor while straight chain monocarboxylic acids were poorly incorporated. The CoA derivatives of acetic and palmitic acids did not act as potential side chain donors and it is significant that the corresponding biosynthetic penicillins are not known. Thiol esters of CoA with acids di-substituted at carbon 2 showed only very weak activity and S-benzoyl CoA was completely inactive (Table I). The enzyme did not appear to be a general acyltransferase. 6-Aminopenicillanic acid was the most active acceptor but splitting the β-lactam ring to give penicic acid with a free carboxyl group destroyed acceptor activity. When the α-carboxyl group was blocked, as the amide or hydrox-

amate, or removed by decarboxylation, appreciable activity was restored. 7-Amino-cephalosporanic acid and its deacetylated derivative, penicillamine and a number of amino acids and peptides were inactive (Table I). The close correlation between the acyl group specificity of the transferase and the production of penicillins in culture (Mortimer and Johnson, 1952) suggests that the enzyme plays a fundamental role in penicillin production. The synthesis of S-phenylacetyl CoA remains to be demonstrated but the CoA derivatives of short chain fatty acids are known and these could be the precursors of the side chains of the natural penicillins.

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REFERENCES

Batchelor, F.R., Doyle, F.P., Nayler, J.H.C. and Robinson, G.N. (1959) Nature 183, 257.

Demain, A.L. (1966) in "Biosynthesis of Antibiotics," J.F. Snell, ed. Vol. I, p. 29, Academic Press, New York.

Kornberg, A., Kornberg, S.R. and Simms, E.S. (1956) Biochim. Biophys. Acta 20, 215.

Lipmann, F. and Tuttle, L.C. (1945) J. Biol. Chem. 159, 21.

Moldave, K. and Meister, A. (1957) J. Biol. Chem. 229, 463.

Mortimer, D.C. and Johnson, M.J. (1952) J. Amer. Chem. Soc. 74, 4098.

Peterson, W.H. and Wideburg, N.E. (1960) Proc. 4th Intern. Congr. Biochem. Vienna 15, 136.

Pruess, D.L. and Johnson, M.J. (1965) J. Bacteriol. 90, 380.

Pruess, D.L. and Johnson, M.J. (1967) J. Bacteriol. 94, 1502.

Stadtman, E.R. (1957) in "Methods in Enzymology," S.P. Colowick and N.O. Kaplan, eds., Vol. III, p. 931, Academic Press, New York.

Tardrew, P.L. and Johnson, M.J. (1959) J. Biol. Chem. 234, 1850.

Wolff, E.C. and Arnstein, H.R.V. (1960) Biochem. J. 76, 375.