V. BIOSYNTHESIS OF BENZYLPENICILLIN (G), PHENOXYMETHYLPENICILLIN (V) AND OCTANOYLPENICILLIN (K) FROM GLUTATHIONE *S*-DERIVATIVES

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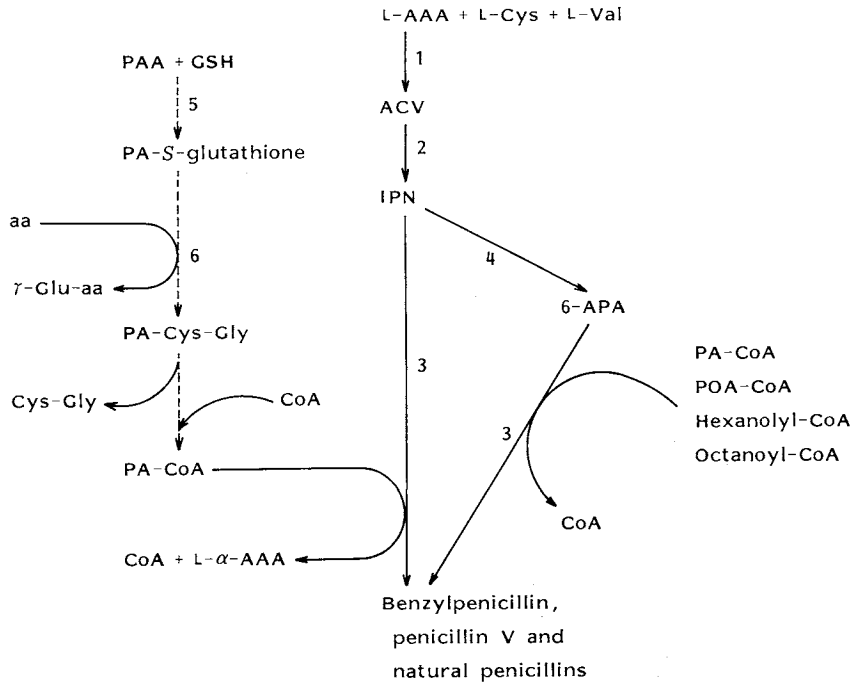
"*In vitro*" synthesis of benzylpenicillin and phenoxymethylpenicillin has been carried out by direct *N*-acylation of 6-aminopenicillanic acid (6-APA) with *S*-phenylacetyl- and (*S*-phenoxyacetyl)glutathione. The reactions were catalyzed by the enzyme acyl-CoA: 6-APA acyltransferase (AT) from *Penicillium chrysogenum* and in both cases the synthesis of antibiotics was enhanced by CoA. Penicillin K, a natural penicillin, was also synthesized "*in vitro*" by incubating (*S*-octanoyl)glutathione, 6-APA and AT, but in this case the formation of antibiotic required the presence of CoA. Furthermore, benzylpenicillin was obtained from (*S*-phenylacetyl)cysteinylglycine and 6-APA, suggesting that some intermediates of the γ-glutamyl cycle are directly involved in the biosynthesis of penicillins.

To explain "*in vivo*" formation of this β-lactam antibiotic, a biosynthetic pathway which includes several glutathione-*S*-derivatives and a non-enzymatic reaction, is proposed.

The enzyme Acyl-CoA: 6-Aminopenicillanic acid acyltransferase (AT) of *Penicillium chrysogenum* catalyzes the synthesis of various penicillins when 6-aminopenicillanic acid (6-APA) is incubated with different acyl-CoA derivatives[1~3]. AT accepts as substrates hexanoyl, heptanoyl, octanoyl, phenylacetyl and phenoxyacetyl-CoA, suggesting that "*in vivo*" a single enzyme is able to synthesize benzylpenicillin, phenoxymethylpenicillin and natural penicillins (F, DF and K)[2] (Fig. 1). Further knowledge of the specificity of this enzyme has been aquired by testing several variants of the phenylacetyl-CoA[3]. We have reported that AT recognized as substrates different acyl-CoA derivatives (R-CO~*S*-CoA) provided that an acetyl-CoA moiety is present in their structure[3]. Although the phenyl ring could be replaced by thiophene (2-thiopheneacetyl-CoA), other modifications of the ring (indole acetyl-CoA) or of the acyl-moiety (benzoyl-CoA, phenylpropionyl-CoA, phenylbutyryl-CoA or phenylglycyl-CoA) were not tolerated by the enzyme[3].

Until now there has been no information about whether AT is able to use as substrates other kinds of thiol esters different from R-CO~*S*-CoA. To shed some light on this point, we have synthesized a variety of *S*-aryl- and (*S*-alkyl)glutathione derivatives and tested their ability to serve as penicillin side chain precursors. In the present article we describe, for the first time, the "*in vitro*" biosynthesis of benzylpenicillin and phenoxymethylpenicillin from 6-APA and (*S*-aryl)glutathione derivatives, reporting a new function for glutathione (GSH) and reinforcing the metabolic importance of this tripeptide. We also give experimental support for a benzylpenicillin biosynthetic model which includes several glutathione-*S*-derivatives and a non-enzymatic step as being necessary for the synthesis of phenylacetyl-CoA. Finally, we describe a hydrolytic activity for acyltransferase which would be responsible for the enzymatic breakdown of the substrate (phenylacetyl-CoA) generating, in the absence of 6-APA, free phenylacetic acid and CoA.

Fig. 1. Postulated branched pathway of penicillin biosynthesis in *Penicillium chrysogenum*.



L-α-AAA, L-α-aminoadipic acid; ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; IPN, isopenicillin N; 6-APA, 6-aminopenicillanic acid; PAA, phenylacetic acid; aa, amino acid; 1, L-α-aminoadipyl-L-cysteinyl-D-valine synthetase (ACV synthetase); 2, isopenicillin N synthase (ACV cyclase); 3, acyl-CoA: 6-APA acyltransferase; 4, isopenicillin N amidolyase; 5, glutathione *S*-transferase; 6, γ-glutamyl-transpeptidase. Postulated metabolic steps are indicated with dashed lines.

Materials and Methods

Materials

Benzylpenicillin potassium salt (1,590 U/mg), 6-APA and *Escherichia coli* penicillin acylase were a gift from Antibióticos, S.A. (León, Spain). β-Lactamase from *Bacillus cereus* was obtained from Difco (U.S.A.). Phenoxymethylpenicillin (1,520 U/mg) was from Marsing and Co., Ltd. (Denmark). L-Cysteinyl-L-glycine was purchased by Bachem Feinchemikalien AG (Switzerland). All other products were of analytical quality or HPLC grade.

Microorganisms

P. chrysogenum Wis 54-1255 (ATCC 28089) was obtained from the American Type Culture Collection. This fungus was used for obtaining pure AT as previously described[3]. Penicillin fermentations were carried out as reported[4,5].

Micrococcus luteus (ATCC 9341) was used as test microorganism for the bioassay of penicillins[6]. Strains were maintained in the lyophilized state.

GS~CO-R Synthesis

(*S*-Phenylacetyl)-, (*S*-phenoxyacetyl)- and (*S*-octanoyl)glutathione were synthesized by standard procedures using the acyl chloride derivative and GSH[3]. A solution of 3.25×10^{-4} mol of the acyl chloride in 1 ml of ether was added dropwise to an ice-cooled solution of 100 mg (3.25×10^{-4} mol) of GSH in 3 ml of aqueous 0.6 M $KHCO_3$ (adjusted to pH 7.5) and the mixture was vigorously stirred for 30 minutes. The pH of the reaction was maintained between 7.0~7.2 with 0.1 M KOH. Under these conditions the amino terminal group of GSH is mainly present as NH_3^+ ($pKa = 9.65$)[7]. The reactions were stopped at 30 minutes extracted with 2 × 5 ml of diethyl ether and the organic and aqueous phases were separated. All the aqueous

solutions were mixed, frozen and later lyophilized. The efficiency of conversion was evaluated using a quantitative assay of free-SH as previously described[3]. The yields of transformation (acyl-GSH formation) were higher than 89% in all cases studied. (*S*-Phenylacetyl)cysteinylglycine was obtained by the same procedure. When required, penylacetyl, phenoxyacetyl- and octanoyl-CoA were also synthesized using the acyl chloride and CoA[3].

The reaction products were further purified using a preparative HPLC column (see below).

Enzymatic Synthesis of Penicillins from GS~CO-R

At a total volume of 85 μl the reaction mixture contained the following: TC buffer (0.05 M Tris-HCl, 0.1 M NaCl) 50 μl; 10 mM phenylacetyl-CoA (or the corresponding GSH *S*-acyl derivative) 10 μl; 0.3 mM 6-APA, 10 μl; 2 mM dithiothreitol (DTT), 5 μl, pure acyltransferase 10 μl (4 μg of protein). When required 10 μl of 20 mM CoA or 10 μl of 10 mM (*S*-phenylacetyl)cysteine were added to the reactions. Incubations were carried out at 25°C for 30 minutes (or the required time) and stopped by adding 85 μl or pure methanol[1~3]. The antibacterial activity of the penicillins produced was estimated by bioassay against *M. luteus* using benzylpenicillin as a standard. Analysis of the reaction products was also monitored by HPLC as previously described[2,8].

HPLC Equipment and Chromatographic Procedure

HPLC (Spectra-Physics SP 8800) equipped with a variable wavelength UV/VIS detector (SP 8450), a computing integrator (SP 4290) and a microparticulate reversed phase (RP-8) column (Spheri-5, 220 × 4.6 mm i.d.) (Brownlee Laboratories) was used.

The mobile phase was 0.2 M phosphate (pH 4.5) and 2-propanol (93:7). The flow-rate was set at 1.5 ml/minute and the eluate was monitored at 254 nm.

For the purification of the GS~CO-R derivatives a preparative column packed with Nucleosil 7 C8 (25 cm × 10 mm i.d.) was used.

Results and Discussion

Some time ago it was suggested that the tripeptide GSH or GSH analogues might play an important role in the biosynthesis of penicillins[9,10]. Participation of GSH is supported by the following observations: (a) GSH has a similar structure to δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), the first intermediate of the biosynthetic pathway common to many β-lactam antibiotics[11~14]. (b) GSH was utilized in preference to inorganic sulfate as source of penicillin sulfur by *P. chrysogenum*[15] and (c) GSH is an inhibitor of isopenicillin N synthase[16], the key enzyme involved in the biosynthesis of β-lactam antibiotics derived from ACV. However, until now no evidence pointing to a direct participation of GSH or GSH-*S*-derivatives in the biosynthetic pathway of penicillins has been found. In exploring the possibility of a direct involvement, we have studied the enzymatic transformation of several (*S*-aryl)- and (*S*-alkyl)glutathione derivatives into penicillins. Incubation of AT from *P. chrysogenum* with 6-APA and different (*S*-acyl)glutathione generated molecules active against *M. luteus* when (*S*-phenylacetyl)- and (*S*-phenoxyacetyl)glutathione were used as substrates but not with (*S*-octanoyl)glutathione (Figs. 2a~2d). Addition of CoA to the reaction mixtures strongly stimulated the biosynthesis of benzylpenicillin and phenoxymethylpenicillin (Figs. 2c and 2d) and, surprisingly, allowed the synthesis of octanoylpenicillin (K) (Fig. 2b). However, CoA did not increase the enzymatic synthesis of benzylpenicillin when phenylacetyl-CoA was used as substrate (Fig. 2a) suggesting that CoA is not a positive effector of AT. This stimulatory effect will be further discussed below.

In standard reactions in which R-CO~*S*-glutathione or 6-APA were omitted no synthesis of antibiotic took place (Fig. 2). All the reaction products were completely inactivated by the enzymes β-lactamase and *E. coli* penicillin acylase (except penicillin K which is less sensitive to the attack of this latter enzyme),

indicating that they are β-lactam antibiotics with an aromatic side chain. The products were purified[17] and further identified by HPLC[2,8] as benzyl, phenoxymethyl and octanoylpenicillin (G, V and K).

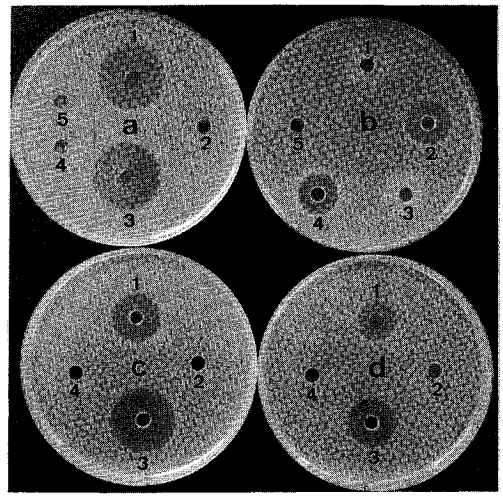
Although the above data demonstrated that glutathione-*S*-derivatives can be used "*in vitro*" for the enzymatic synthesis of different penicillins, they do not establish whether *P. chrysogenum* can obtain "*in vivo*", through the γ-glutamyl cycle, metabolic intermediates directly involved in the biosynthesis of penicillins. To obtain data bearing on this possibility, we investigated the matter studying the following:

a) The enzymatic acylation of 6-APA using AT and (*S*-phenylacetyl)derivatives of some molecules directly related to the γ-glutamyl cycle.

b) The stimulation of penicillin biosynthesis observed in the presence of CoA when (*S*-aryl)- and (*S*-alkyl)glutathione were tested as substrates of AT.

Glutathione *S*-transferases catalyze reactions in which GSH provides electrons through its sulfur atoms for nucleophilic attack on, or reduction of, a second electrophilic substrate resulting in the formation of a conjugate. The nature of the second substrate can be broad, which explains the central role of these enzymes in detoxification of harmful products (aliphatic, aromatic, heterocyclic compounds, carcinogens, antibiotics *etc.*)[18,19]. The γ-glutamyl moiety of such conjugates is removed by the enzyme γ-glutamyltranspeptidase[20,21] and later the *S*-substituted cysteinylglycines are cleaved by a specific dipeptidase[21] to yield the corresponding *S*-substituted cysteines.

Fig. 2. Bioassay against *Micrococcus luteus* of incubation mixtures containing pure acyl-CoA: 6-APA acyltransferase from *Penicillium chrysogenum*, 6-APA and various other substrates.



(a) 1, Control (1 mM phenylacetyl-CoA); 2, idem incubated with β-lactamase from *Bacillus cereus*; 3, idem 1 but with 2 mM CoA; 4, idem 1 but incubated with *Escherichia coli* penicillin acylase (4 IU/ml); 5, control without phenylacetyl-CoA. (b) 1, control [1 mM (*S*-octanoyl)glutathione]; 2, idem 1 but with 2 mM CoA; 3, idem 2 but incubated with β-lactamase; 4, idem 2 but incubated with *E. coli* penicillin acylase (4 IU/ml) 5, idem 2 but without (*S*-octanoyl)glutathione. (c) 1, control [1 mM (*S*-phenylacetyl)glutathione]; 2, incubated with β-lactamase; 3, idem 1 but with 2 mM CoA; 4, idem 1 but incubated with *E. coli* penicillin acylase (4 IU/ml). (d) 1, control [1 mM (*S*-phenoxyacetyl)glutathione]; 2, incubated with β-lactamase; 3, idem 1 but with 2 mM CoA; 4, idem 1 but incubated with *E. coli* penicillin acylase (4 IU/ml).

We suggest that "*in vivo*" *P. chrysogenum* takes up some penicillin side chain precursors (phenylacetic and phenoxyacetic acids) by binding them to GSH through a reaction similar to that catalyzed by glutathione *S*-transferase (see Fig. 1). The action of γ-glutamyltranspeptidase then gives a product, (*S*-phenylacetyl)cysteinylglycine (PA-cysgly) which could be used as a substrate by AT with the same or higher efficiency than other (*S*-acyl)glutathiones. Fig. 3 shows that incubation of PA-cysgly with AT and 6-APA generated an antibiotic substance sensitive to β-lactamase and *E. coli* penicillin acylase; and this was further identified by HPLC as benzylpenicillin (Fig. 5d). As expected, the quantity of benzylpenicillin produced "*in vitro*" was greater than that obtained with (*S*-aryl)glutathiones (Fig. 2), suggesting that acylation of 6-APA is carried out more efficiently in the presence of PA-cysgly (Fig. 3).

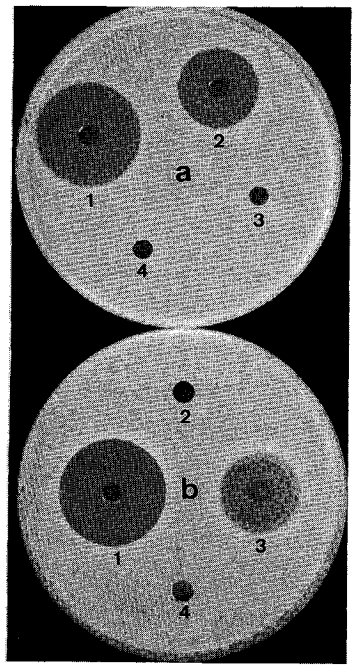
The importance of the glycyl moiety in the recognition of PA-cysgly by AT was established by testing (*S*-phenylacetyl)cysteine (PA-cys) as a potential penicillin side-chain precursor. Fig. 3 shows that PA-cys

is not accepted as a substrate by AT but also inhibits the formation of benzylpenicillin (60%) when supplied to reaction mixtures containing phenylacetyl-CoA and 6-APA. To ascertain whether the lower production of benzylpenicillin observed when PA-CoA was incubated with AT and PA-cys was a secondary effect due to the presence of cysteine in the samples, 1 mM cysteine was added to similar reaction mixtures. In all the cases tested the quantity of benzylpenicillin produced was identical suggesting that at this concentration cysteine does not destroy the penicillin generated by AT (data not shown). These results are consistent with the above hypothesis that PA-cysgly is a true intermediate in benzylpenicillin biosynthesis.

In the second approach, we have studied the stimulatory effect of CoA on the biosynthesis of penicillin "*in vitro*" when *S*-alkyl- (*S*-octanoyl-) or *S*-aryl- (*S*-phenylacetyl-, *S*-phenoxyacetyl)glutathione derivatives were used as substrates of acyltransferase.

It has been reported that some *S*-substituted GSH derivatives directly related to the γ-glutamyl cycle may be formed non-enzymatically by reaction of GSH with certain electrophilic compounds[21]. Therefore, we investigated whether the stimulation caused by CoA might be explained by such a mechanism. The incubation of (*S*-phenylacetyl)cysteinylglycine with CoA and Tris-HCl buffer (without enzyme) generated a large amount of phenylacetyl-CoA (Fig. 4A), whereas in the presence of acyltransferase this molecule almost disappeared (Fig. 4B). To clarify this, AT was incubated with phenylacetyl-CoA for different times. Fig. 5 shows that the pure enzyme almost completely hydrolyzed its natural substrate (phenylacetyl-CoA) in 20 minutes, releasing phenylacetic acid and CoA. However, in the presence of 6-APA phenylacetic acid was not found since it was quickly bound to 6-APA, forming benzylpenicillin (Fig. 5D). These findings are consistent with the suggestions of SPENCER and MAUNG[22] that phenylacetyl-CoA hydrolase is only one of four activities attributable to a single thiol-dependent enzyme that function in a ping-pong mechanism with an alternate hydrolytic step.

Fig. 3. Bioassay against *Micrococcus luteus* when pure acyl-CoA: 6-APA acyltransferase was incubated with 6-APA and different substrates.



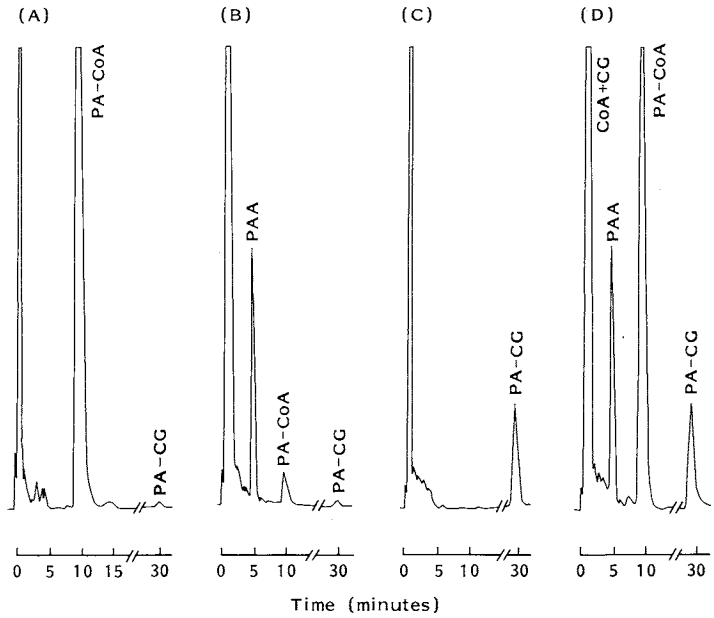
(a) 1, Control [1 mM (*S*-phenylacetyl)cysteinylglycine] and 2 mM CoA; 2, idem 1 but without CoA; 3, idem 2 incubated with β-lactamase; 4, idem 2 incubated with *Escherichia coli* penicillin acylase (4 IU/ml). (b) 1, control (1 mM phenylacetyl-CoA); 2, reaction in which the substrate was (*S*-phenylacetyl)cysteine (1 mM); 3, idem 1 but supplied with 1 mM (*S*-phenylacetyl)cysteine; 4, idem 1 but without 6-APA.

Concluding Remarks

From the above results some conclusions may be drawn:

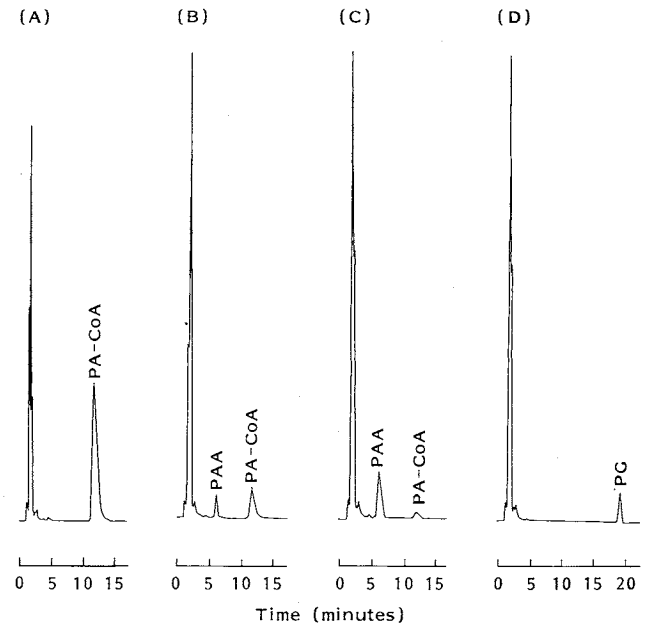
1) Phenylacetyl-CoA is non-enzymatically produced when CoA is incubated with different glutathione-*S*-substituted derivatives. It seems probable that these reactions represent an "*in vitro*" model of the biosynthetic pathway that occur "*in vivo*", since a high intracellular concentration of GSH[23] and

Fig. 4. HPLC chromatograms of the reaction products obtained after incubating at 25°C, 20 minutes.



(A) (*S*-Phenylacetyl)cysteinylglycine (5 mM) and CoA (5 mM), (B) idem A but with acyl-CoA: 6-APA acyltransferase, (C) idem A but without CoA, (D) controls: CoA, coenzyme A; CG, cysteinylglycine; PAA, phenylacetic acid; PA-CoA, phenylacetyl-CoA; PA-CG, (*S*-phenylacetyl)cysteinylglycine.

Fig. 5. HPLC chromatograms of the reactions products obtained after incubating phenylacetyl-CoA (1 mM) and acyl-CoA: 6-APA acyltransferase.



(A) 0 minute, (B) 10 minutes, (C) 20 minutes, (D) idem C but incubated in the presence of 6-APA (30 μM). PAA, phenylacetic acid; PA-CoA, phenylacetyl-CoA; PG, benzylpenicillin.

CoA could facilitate the non-enzymatic formation of phenylacetyl-CoA (see Fig. 1). This reaction could take place by a rapid $S \rightarrow S$ intermolecular acyl migration between the phenylacetyl moieties of (*S*-phenylacetyl)glutathione or (*S*-phenylacetyl)cysteinylglycine and CoA.

2) In the absence of 6-APA, acyl-CoA: 6-APA acyltransferase catalyzes the hydrolysis of phenylacetyl-CoA to its free components.

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