REVIEW ARTICLE

Enzymatic Synthesis of Hydrophobic Penicillins

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I. Summary

Our knowledge of the enzymes and genes involved in the biosynthesis of β-lactam antibiotics has increased notably in the last decade. The purification to homogeneity of some of these proteins as well as their biochemical characterization has allowed some of them to be used for synthesizing many different penicillins and cephalosporin-like products *in vitro*. In this report we describe the most important advances in this field, placing special emphasis on the enzymatic synthesis of hydrophobic penicillins. The use of purified acyl-CoA: 6-aminopenicillanic acid (6-APA) acyltransferase (AT) from *Penicillium chrysogenum* and several acyl-CoA ligases obtained from different microbial origins has led to the reproduction "*in vitro*" of the last step involved in penicillin biosynthesis. By coupling these enzymatic systems (AT and acyl-CoA ligases) an impressive number of β-lactam antibiotics has been obtained. Thus, most of the known natural penicillins, many of the semisynthetic variants and others, which until now can only be obtained chemically, have been synthesized enzymatically from their natural precursors. Furthermore, the use of heterologous proteins in coupled systems has opened a new and exciting field in β-lactam antibiotic research, lending new perspectives to the traditional methodology

This review is dedicated to Prof. A. L. DEMAIN (M.I.T.) who is the master of a generation of scientists and to the memory of a gentleman, Dr. FERNANDS HOLGUERA y Diez de la Lastra, who introduce me to the fermentation processes.

followed by antibiotic fermentation industries.

II. General Introduction

β-Lactam antibiotics are natural products chemically characterized by the presence in their molecule of a four-membered ring (commonly known as the β-lactam ring) and biologically by their antibacterial activity[1~4]. These compounds, which have been classified in different families according to their chemical structure (nocardicins, monobactams, penicillins, clavulanic acid, carbapenems, cephalosporins and cephamycins)[5~11] (Fig. 1), are produced by several microorganisms which may or may not phylogenetically related. Most of these antibiotics are synthesized by bacteria, especially by certain species of *Streptomyces*, as well as by other prokaryotes included in the genus *Nocardia*, *Lysobacter* and *Flavobacterium*. Eukaryotic microbes are also able to synthesize these products, *Emericellopsis glabra*, *Paecylomyces persicinus*, *Aspergillus nidulans*, *Acremonium chrysogenum* and *Penicillium chrysogenum* (particularly the latter two) being the most important producer species[12~14].

Thus, penicillins are a special group of β-lactam antibiotics containing in their molecule two fused rings, the β-lactam and the thiazolidine rings, specific to this family, as well as a variable side chain which characterizes each particular penicillin (Fig. 2). Depending on the structure of the side-chain, these penicillins are included in two different groups: hydrophobic penicillins (those containing a non-polar side chain) and hydrophilic penicillins (with polar side chains) (see Table 1). Bacterial penicillins are hydrophilic whereas fungal penicillins may be hydrophobic or hydrophilic[7]. Hydrophilic penicillins are biosynthetic intermediates which are not usually released into the culture broths, whereas hydrophobic penicillins are final-biosynthetic products which: (i) have as an immediate precursor some hydrophilic penicillins, and (ii) are released and accumulated in the fermentation broths[7,12].

Both kinds of penicillins also have an additional biological difference: their antibacterial activity. Whereas hydrophobic penicillins have a high and broad antibacterial spectrum, hydrophilic penicillins have only restricted antibiotic potency[7,15]. This biosynthetic difference, caused by the existence in the producer microbes of an enzyme which catalyzes the substitution of the isopenicillin N side-chain by other molecules, confers the producer organism with certain ecological advantages[16~18]. For this reason, it is surprising that during the course of evolution prokaryotic microorganisms, which are able to produce hydrophilic but not hydrophobic penicillins, have been unable to develop or capture (by horizontal gene transference for example) this kind of genetic information[19~21]. The fact that only eukaryotic organisms are able to produce hydrophobic penicillins can be explained by assuming that these penicillins, unlike the hydrophilic type, must be extremely toxic, or even lethal, for bacterial strains. Several interesting reviews about the role of secondary metabolites have been published recently[22~24].

III. Penicillin Biosynthetic Pathway

The biosynthetic pathway of the most important β-lactam antibiotics (penicillins, cephalosporins and cephamycins) (Fig. 3) is a partially common route starting with the non-ribosomal condensation of the free amino acids L-α-aminoadipic acid, L-cysteine and L-

Fig. 1. Structure of the different families of naturally occurring β-lactam antiobiotics.

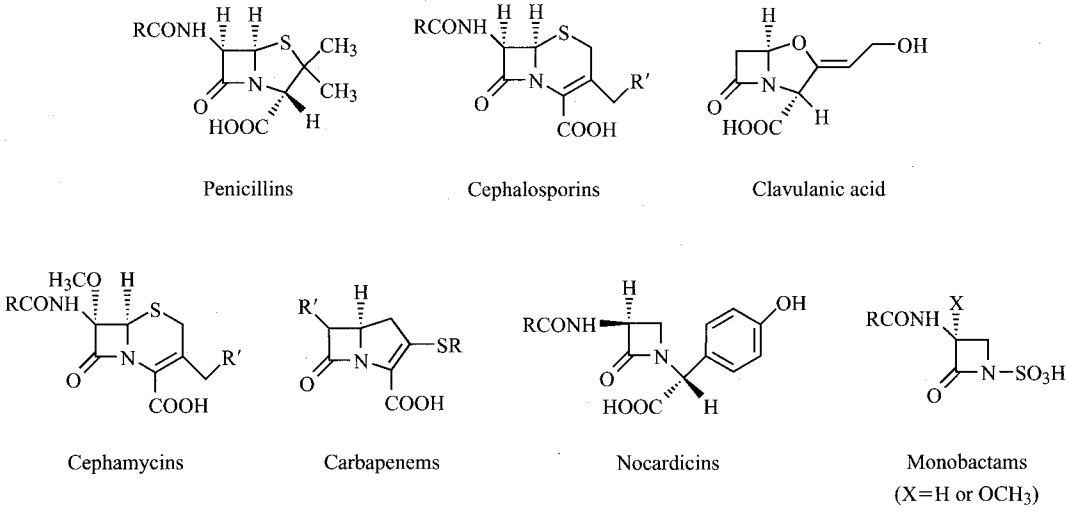
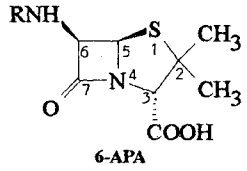


Fig. 2. Structure of 6-aminopenicillanic acid (6-APA) and different penicillins.



6-APA

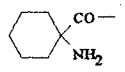
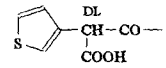
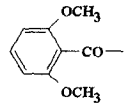
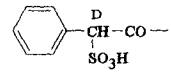
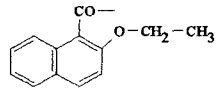
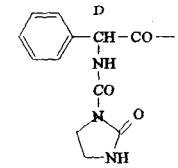
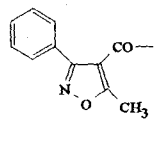
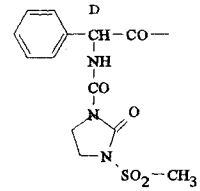
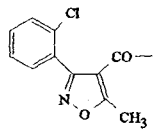
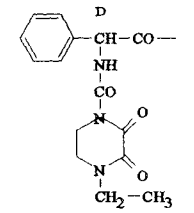
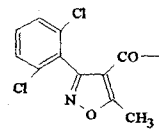
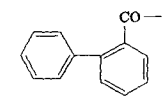
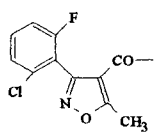
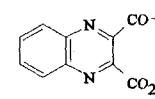
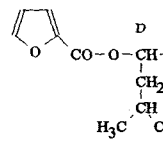
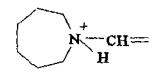
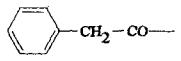
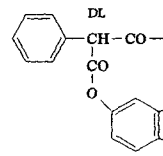
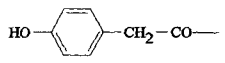
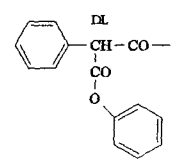
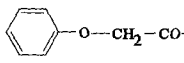
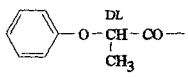
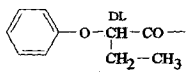
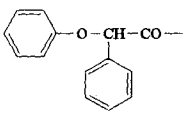
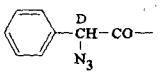
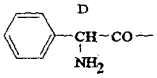
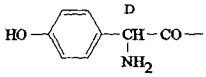
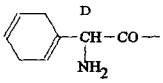
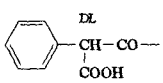
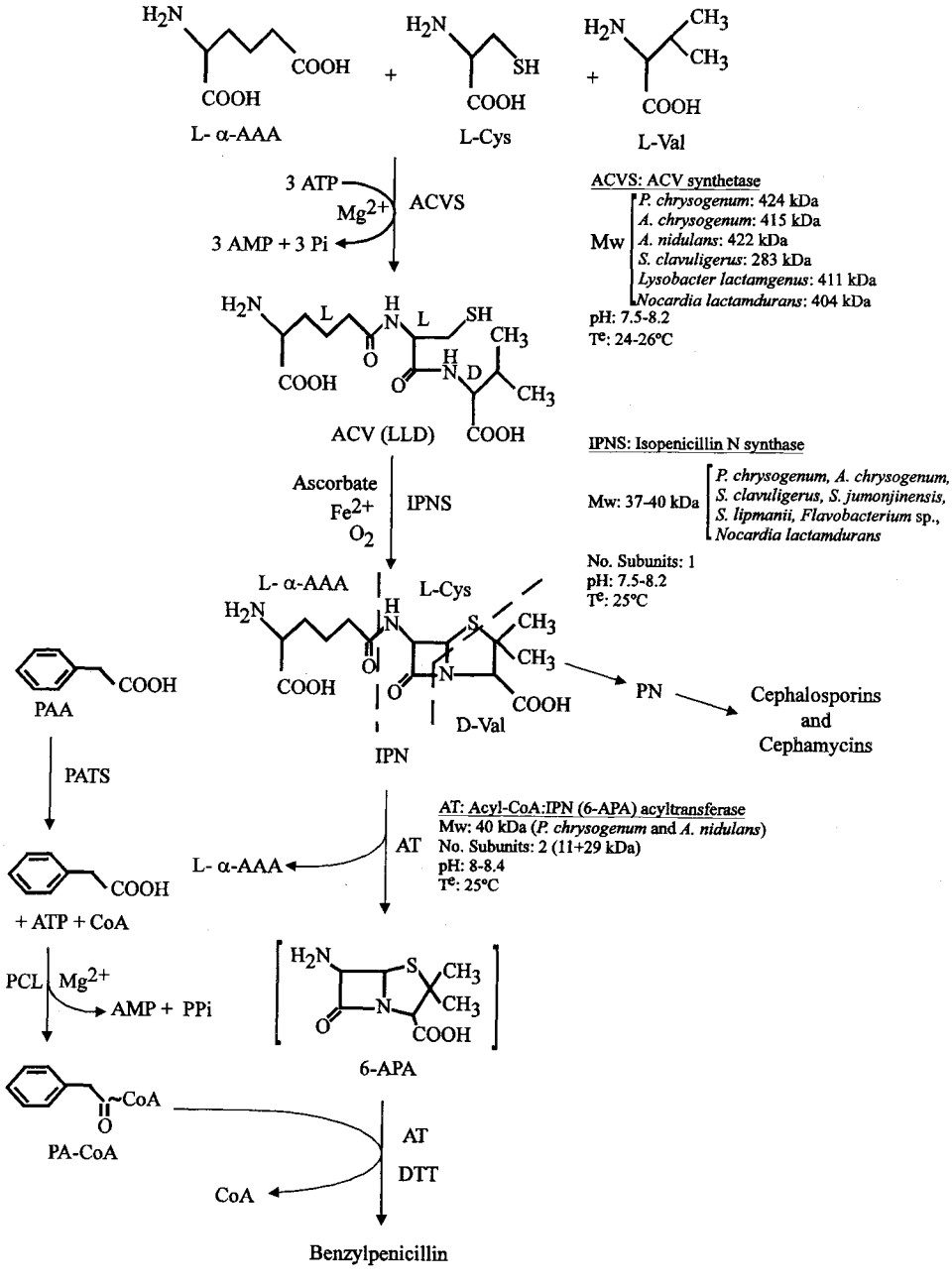
R	Penicillin	R	Penicillin	R	Penicillin
$CH_3-(CH_2)_4-CO-$	DF		Ciclacillin		Ticarcillin
$CH_3-CH_2-CH=CH-CH_2-CO-$	F		Methicillin		Sulbenicillin
$CH_3-(CH_2)_6-CO-$	K		Nafcillin		Azlocillin
$CH_3-(CH_2)_3-S-CH_2-CO-$	BT		Oxacillin		Mezlocillin
$CH_2=CH-CH_2-S-CH_2-CO-$	O		Cloxacillin		Piperacillin
$CH_3-\underset{Cl}{C}=CH-CH_2-S-CH_2-CO-$	S		Dicloxacillin		Diphenicillin
$H_2N-\underset{COOH}{\overset{L}{CH}}-(CH_2)_3-CO-$	IPN		Floxacillin		Quinacillin
$H_2N-\underset{COOH}{\overset{D}{CH}}-(CH_2)_3-CO-$	N		Furbucillin		Mecillinam
	G		Carindacillin		
	X		Carfecillin		
	V				
	Phenethicillin				
	Propicillin				
	Phenbenicillin				
	Azidocillin				
	Ampicillin				
	Amoxicillin				
	Epicillin				
	Carbenicillin				

Table 1. Classification of penicilins.

A.	Regarding their chemical structure
	• Hydrophobic: With non-polar side chain (DF, F, K, BT, O, S)
	• Hydrophilic: With polar side chain (IPN, PN, 6-APA)
B.	Regarding their biosynthetic origin
	• Naturally occurring: R (side chain) can be obtained through the general metabolism (DF, F, K, BT, O, S, IPN, PN, 6-APA)
	• Biosynthetic: R must be added to the cultures (G, V, X)
	• Semi-synthetic: R can only be attached by chemical synthesis (ampicilin, amoxicilin, methicillin, *etc.*).

valine in a linear tripeptide molecule (L-α-aminodipyl-L-cysteinyl-D-valine, ACV) lacking antibacterial activity[25~27]. This transformation is catalyzed by the enzyme ACV synthetase[28,29]. In a second step, the moieties of L-cysteine and D-valine of ACV are cyclized to the β-lactam and thiazolidine rings (common for all the penicillins), L-α-aminoadipic acid remaining as a side-chain. This molecule, which is the first compound of the pathway with antibacterial activity, is named isopenicillin N (IPN) and the catalyzing enzyme, IPN synthase[30~32]. IPNS, probably the best known protein of the penicillin biosynthetic pathway, was the first enzyme of the route purified to homogeneity and whose

Fig. 3. Biosynthetic pathway of penicillin and other β-lactam antibiotics.



gene has been cloned, sequenced and expressed in different microorganisms[32~35]. IPN is the last common intermediate of the penicillin and cephalosporin (or cephamycin) pathway (Fig. 3). Thus, whereas in the biosynthetic pathway of cephalosporin and cephamycins, IPN is transformed to penicillin N (a compound similar to IPN in which L-α-aminoadipic acid is racemized to D-α-aminoadipic acid) by an epimerase or racemase[14], in the fungi *P. chrysogenum* and *A. nidulans* a different enzyme—acyl-CoA : 6-aminopenicillanic acid acyltransferase (AT)—catalyzes the exchange of the L-α-aminoadipic moiety with other side-chains, thus generating different final products (see below). The different reactions included in the penicillin biosynthetic pathway and the most important characteristics of the enzymes involved in it are summarized in Fig. 3.

IV. Pioneering Studies on the Enzymatic Synthesis of Penicillins: How They Arise

The most important microbes able to synthesize penicillins and cephalosporins are *P. chrysogenum* and *A. chrysogenum*. Using empirical mutation procedures[16,36], overproducer mutants—which in submerged cultures reach high benzylpenicillin (about 57 g/liter) or cephalosporin (22 g/liter) titres—have been isolated. However, although considerable success has been achieved in the isolation of mutants and in the determination of the optimal conditions for their industrial production, the molecular bases of benzylpenicillin and cephalosporin biosynthesis have only been approached recently (see below). The restricted biosynthetic capacity in some cases, the appearance of bacterial resistances to these antibiotics (penicillinases and cephalosporinases) in others, as well as limitations in the improvement of production due to the complex engineering problems in industrial tanks have prompted workers to seek solutions for these problems through molecular knowledge of the enzymatic steps involved in penicillin biosynthesis[12,18,30~32]. It is therefore clear that pioneering studies on the "*in vitro*" synthesis of penicillins would arise as an immediate consequence of the need to characterize some of the physicochemical properties, kinetic parameters and substrate specificities of the enzymes involved in their biosynthetic pathways. The transformation of ACV into IPN observed by ABRAHAM's group at Oxford and DEMAIN and coworkers at MIT[37,38] was the first demonstration that it was possibly transformed *in vitro* molecules without antibacterial activity into penicillins. Over a relatively short period of time, the good collaboration between chemists, biochemists and microbiologists allowed a considerable advance in the knowledge of the enzymes' structures as well as of the mechanisms of the reactions catalyzed by them. A good example of this kind of research is the excellent work performed with IPNS[30,31]. BALDWIN and coworkers at Dyson Perryns Lab. in Oxford have synthesized an impressive number of ACV analogues, affording these workers an understanding of the molecular bases of the ACV-cyclization mechanism as well as enabling them to chemically reproduce the synthesis of many penicillins *in vitro* (for a review see 12,14,30~31,34). Unfortunately, most of these studies have been carried out with enzymes or enzymatic systems obtained from *A. chrysogenum* or *S. clavuligerus*; although these microbes synthesize hydrophilic penicillins, they are unable to produce hydrophobic penicillins[14,39] (see Table 1). For this reason the *in vitro* synthesis of these latter has only been approached in certain cases[40~44].

V. Enzymes Required for the *In Vivo* Synthesis of Hydrophobic Penicillins

The last step of the biosynthesis of benzylpenicillin (Fig. 3) is catalyzed by at least three different enzymes (or enzymatic systems). In the initial reaction, phenylacetic acid (PAA), the side-chain precursor of benzylpenicillin, must be taken up by *P. chrysogenum* to be incorporated into the molecule of antibiotic. This uptake system is an active transport system (phenylacetic acid transport system, PATS), specifically induced by PAA, which only appears when certain nutrients have been exhausted from the broths[45,46]. PATS is strictly regulated by carbon and nitrogen sources, this being a critical regulatory point which very precisely controls the quantity of penicillin G produced[47,48]. The inhibition of PATS has as a direct consequence, the biosynthesis and accumulation in the broth of other hydrophobic penicillins (F, DF, K) containing a fatty acid as the side-chain precursor which can be obtained, unlike PAA, from an endogenous origin (they do not have to be supplied to the cultures)[49~51] (Fig. 9).

Once the precursor has been taken-up, it must be activated to a CoA thioester (PA-CoA). The enzyme which catalyzes this reaction seems to be a phenylacetyl-CoA ligase (PCL) with narrow substrate specificity[52~53].

Finally, the enzyme acyl-CoA 6-APA (isopenicillin N) acyltransferase (AT) catalyzes either the exchange of the α-aminoadipyl moiety of IPN or the acylation of the amino group of 6-APA with other side chain precursors previously activated to CoA derivatives (Fig. 3)[54~59].

This enzyme, which has only been reported in Plectomycetes[18], has been purified to homogeneity from *P. chrysogenum* and *A. nidulans*[60~62] and the enzymes seem to be similar. AT is a protein which has the following enzymatic activities: a) penicillin acyltransferase; b) 6-APA acyltransferase; c) penicillin acylase and d) phenylacetyl-CoA hydrolase[63~65].

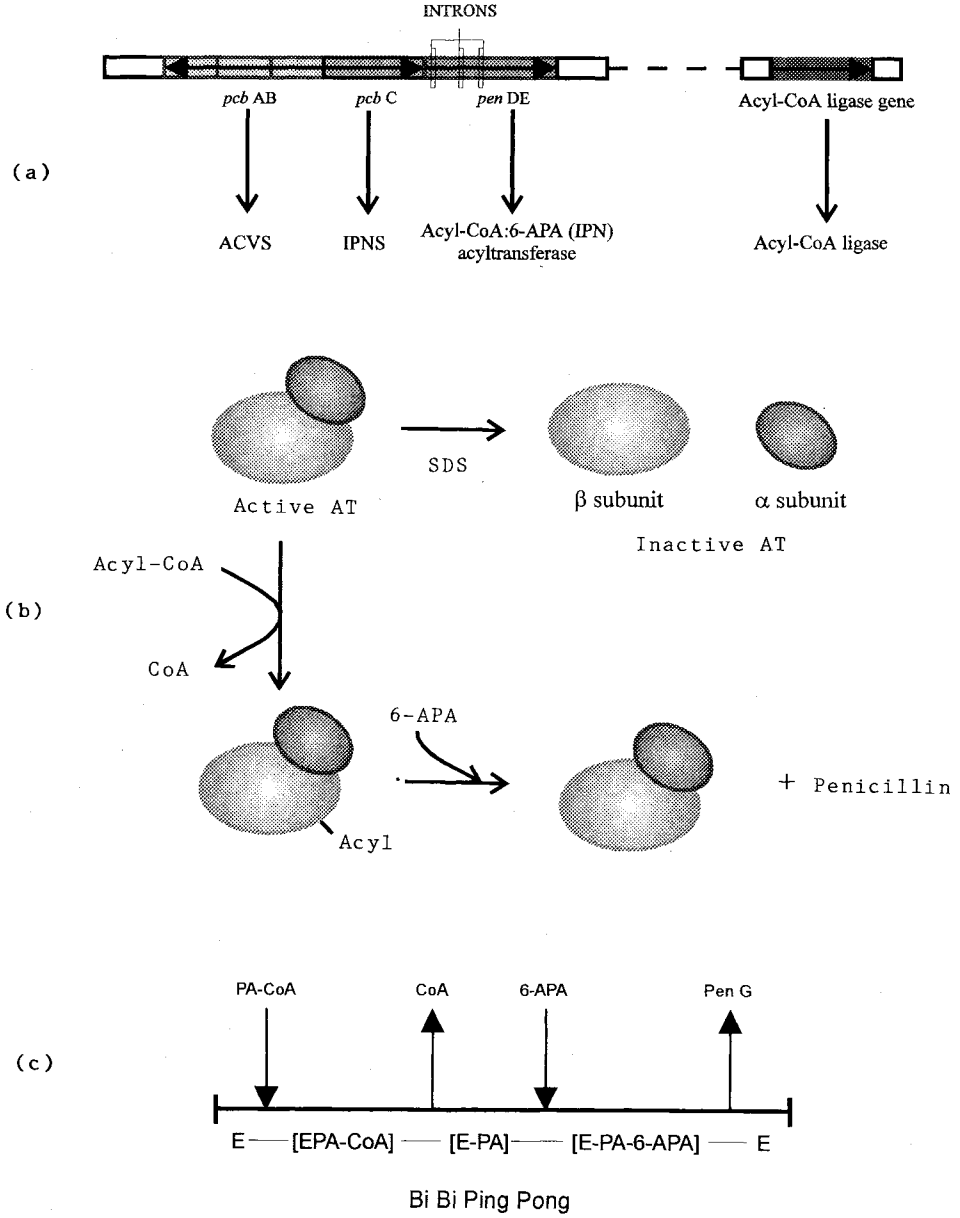
From a structural point of view, AT is a protein (Mr 40 kDa) formed by two different subunits (α, β heterodimer) with a Mr of 11 and 29 kDa, respectively[12,62]. Both subunits are encoded by a single gene undergoing a posttranslational cleavage to the mature functional enzyme[66~69]. Furthermore, these two proteins (α, β) seem to be required to catalyze *in vitro* the *N*-acylation of 6-APA[62]. A molecular approach to the study of the amino acids involved in the activity or in proenzyme cleavage has been recently published by different authors[70,71]. The possible function(s) of these subunits during the catalysis will be discussed later.

The AT gene is located 3′ to the IPNS gene in *P. chrysogenum* and *Aspergillus nidulans* biosynthetic cluster[72~75] (see Fig. 4). It has been sequenced, cloned and expressed in *E. coli*[66~68,76]. The gene which is split by three introns has not been found in prokaryotic microbes, suggesting that it has a eukaryotic orgin[18,67].

VI. Location of Penicillin Biosynthetic Enzymes

The subcellular location of the most important

Fig. 4. Genetic organization of the penicillin biosynthetic cluster in *P. chrysogenum* and *A. nidulans* (a) and schematic representation of the 6-APA acylation mechanism (b and c) proposed by different authors (see text).



enzymes involved in the biosynthesis of penicillins (ACVS, IPNS, AT and PATS) have been approached recently[45,77~80]. Using inmunological techniques, it has been demonstrated that in fungi ACVS is either within or bound to the vacuolar membranes[77,80], whereas IPNS seems to be a cytosolic enzyme[78]. Moreover, AT was found in microbodies[80] suggesting that these organelles could play an important function in the biosynthesis of penicillins. Finally PATS, the system responsible for the uptake of PA, is located in the plasma membrane[45]. The participation of different organelles in a single biosynthetic pathway involves the existence of other, hitherto unknown enzymes (permeases, binding proteins) that catalyze the transference of substrates between different environments (Fig. 9). These results, which suggest a complicated organization for the penicillin biosynthetic pathway[78], could be simplified if some of the penicillin side-chain precursors (fatty acids or their CoA derivatives) were synthesized in the same organelle. The demonstration of β-oxidation systems (and therefore the presence of acyl-CoA derivatives) in catalase-free microbodies[81] strongly suggests a direct role of these organelles in at least the last step of hydrophobic penicillin biosynthesis.

VII. AT Mechanistic Studies

In 1970, SPENCER and MAUNG reported that the four activities attributable to AT could be explained by a Ping Pong Bi Bi mechanism[63]. Unfortunately, further kinetic studies with this enzyme were not reported. Very recently, BALDWIN and coworkers[69] investigated the posttranslational modifications as well as the mechanism of AT using electrospray mass spectrometry. These authors reported that when AT was purified from mycelia grown in media containing a penicillin side-chain precursor, the α-subunit was acylated with a molecule with the same mass of the compound used as precursor; however, in the absence of penicillin side-chain precursor (only 6-APA and natural penicillins can be produced), this subunit was not acylated[69].

Additionally, the incubation *in vitro* of AT (α, β) with different acyl-CoA derivatives showed that acylation was specific for the β but not for the α-subunit. Furthermore, the acyl group linked to the β subunit was lost after incubating the protein with 6-APA, probably through nucleophilic attack[69]. A schematic representation of all these findings and the original hypothesis of SPENCER and MAUNG is summarized in Fig. 4.

The reason for these different *in vivo* and *in vitro* acylating mechanisms remains unclear. It could be speculated that the α-subunit would act as a recipient of exogenously added acyl-penicillin side-chain precursors, whereas the β-subunit would only be acylated with those compounds synthesized by the cells. However, after incubating AT with PA-CoA, a mass shift corresponding to PA incorporation was observed[69]. This indicates that PA, which cannot be synthesized by *P. chrysogenum*, is also able to acylate the β-subunit.

Another possible explanation for these findings[69] could be that the β-subunit would only recognize acyl-CoA variants whereas the α-subunit would accept those not esterified with CoA. The location of AT in microbodies, the description of β-oxidation in such organelles, and the observation that AT is able to accept *S*-glutathione derivatives of PAA as substrates[82], could explain the different acylations observed *in vivo* and *in vitro* in the two subunits. Although further experiments are obviously required to clarify the exact mechanism of acylation, the above experiments have provided a considerable advance in its understanding.

VIII. *In Vitro* Synthesis of Hydrophobic Penicillins

Because the synthesis of benzylpenicillin from PAA *in vivo* requires the participation of two different enzymes, PCL and AT, two different approaches have been followed to reproduce *in vitro* the enzymatic synthesis of hydrophobic penicillins:

A. Study of the substrate specificity of AT.

B. Isolation, purification and characterization of several acyl-CoA synthetases from different microorganisms and study of the optimal physicochemical conditions required for coupling each of these acyl-CoA- activating enzymes and AT *in vitro*.

A. Substrate Specificity of AT

The molecules that can be used as substrates by AT, and therefore be converted into penicillins, were analyzed using different acyl-CoA thioesters (aliphatic or aromatic) or by replacing 6-APA with a chemically related molecule. The reaction scheme was as follows:

$$\text{X-CoA} + \text{6-APA (or analogous)} \xrightarrow[\text{AT}]{\text{DTT}} \text{Penicillin} + \text{CoA}$$

1. X-CoA is an Aliphatic Molecule of Variable Carbon Length

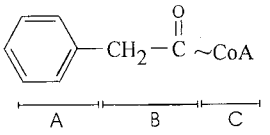
For a long time it was known that several penicillins containing fatty acids as the side-chain with different carbon lengths (hexanoic, 3-hexenoic, or octanoic acid)

were usually produced *in vivo* by *P. chrysogenum* strains[7] and that the rate of synthesis of these penicillins (DF, F and K) increased when the fungus was cultured in the absence of an exogenous penicillin side-chain precursor (*i.e.* in fermentations without either PAA or phenoxyacetic acid –POA–). Accordingly, the first experiments were directed to showing whether AT was the only enzyme responsible for the synthesis of hydrophobic penicillins or whether, by contrast, more than one enzyme would be required to transacylate the α-aminodipic acid moiety of IPNS with other penicillin side-chain precursors. It was observed that AT was very efficient at catalizing the synthesis of penicllins F, DF and K as well as others containing as the side-chain either saturated or unsaturated linear aliphatic molecules with the only requirements that: (i) their carbon length should range between 6 to 8 carbon atoms and (ii) free rotation of the C_2-C_3 bond should exist[49,83]. The substitution in such molecules of a methylene group by a carboxy group or the replacement of a methylene by a keto group notably decreased the rate of conversion into penicillins[85]. Furthermore, when branched or cyclic aliphatic molecules were tested, longer incubation times were usually required to obtain a detectable quantity of antibiotic[84] suggesting that these compounds are poorly used by AT as substrates.

It is interesting to note the good correlation observed between the data obtained by APLIN *et al.*[69] about the acylation of the β-subunit of AT and those previously reported by us about the substrate specificity of this enzyme (see above). With the exception of octanoyl-CoA, for which acyl-enzyme was not observed[69], all the other molecules able to acylate the β subunit have been also reported to be converted into penicillin by AT[12,18,49,83~84]. In a later paper, MARTIN and coworkers[65] obtained similar results; only in some cases (valeryl-CoA and nonanoyl-CoA) are there certain discrepancies with the data reported by other scientists (see above).

2. Use of PA-CoA Variants as Penicillin Side-chain Precursors

The natural substrate of AT (PA-CoA), which is also the direct precursor of benzylpenicillin, was divided in three parts (A, B and C, see below) and different analogues were synthesized by modifying one of them and keeping the other two constant.



Accordingly, the following groups of substrates were tested:

—GROUP A included:

—molecules with substitution of one or more hydrogen atom(s) of the ring by a different atom or groups of atoms (A_1).

—molecules in which the benzene ring was replaced by others (A_2).

—GROUP B included:

—molecules with variations in the carbon length (shorter or longer) of the acyl-moiety (B_1).

—molecules with substitution on the α-carbon atom (B_2).

—GROUP C included:

—substrates in which CoA was replaced by other SH-containing molecules.

i. Substrates are Molecules Included in the A_1 Subgroup

To establish how critical the carbon length of the aromatic chain linked to the acetyl-CoA moiety was, as well as the importance of its molecular size in the formation of the enzyme substrate complex, different phenylacetyl-CoA variants were assayed. Penicillins were only obtained when *o*-, *m*- or *p*-hydroxy; *m*- or *p*-methyl or *m*- or *p*-methoxy; *m*- or *p*-amino and *o*-, *m*- or *p*-fluoro derivatives of PA-CoA were employed. However, the presence of a NO_2 group on the aromatic ring prevented or hindered the use of these molecules as substrates[61,85~88]. Other halogen derivatives tested (Cl- or Br-PA-CoA) were not transformed into penicillins[88].

One could surmise that some of the molecules included in this group would be used as substrates by AT because they mimic rigid structural variants of the aliphatic side-chain present in some natural substrates (hexanoyl- and octanoyl-CoA) (Fig.5). A similar hypothesis was proposed by BALDWIN *et al.* to explain the substrate specificity of IPNS when the α-aminoadipoyl moiety of ACV was replaced by PAA and *m*-carboxy PAA[41]. The results obtained when methyl- and methoxy-PA-CoA variants were used as substrates[85~87] seem to agree with the minimal and maximal side-length requirements re-

Fig. 5. Structure of different acyl-CoA derivatives. A and B correspond to rigid conformations of these molecules.

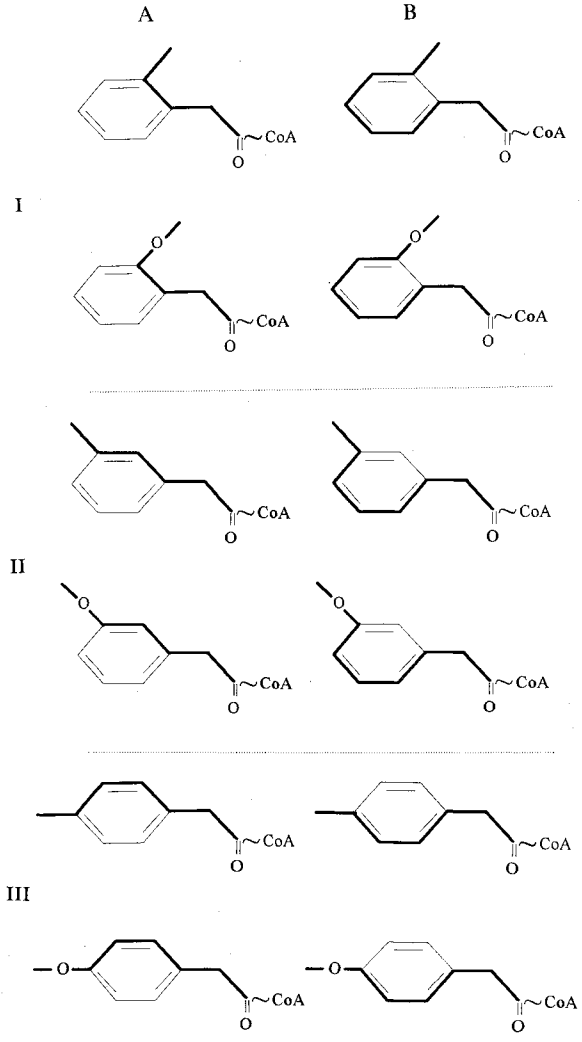
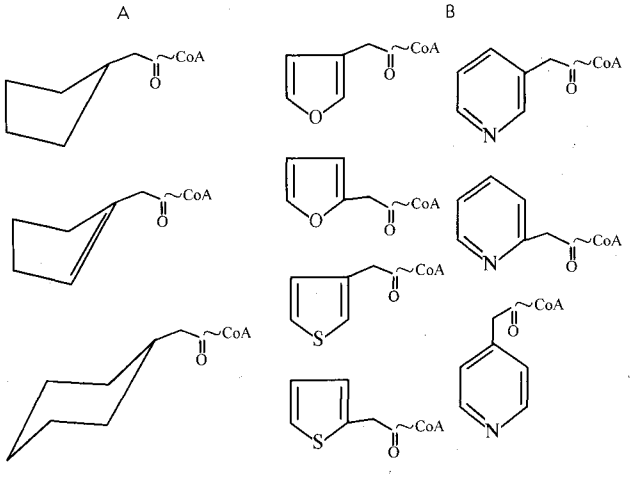


Fig. 6. Structure of different acyl-CoA derivatives obtained by replacing the benzene ring of phenylacetic acid by other rings.



ported for AT[49,61] since *p*- and *m*-methylphenylacetyl-CoA (as well as *p*- and *m*-methoxyphenylacetyl-CoA) could represent rigid structures ranging between 6 to 8 carbon atoms in length. However, in *o*-methyl (or *o*-methoxy) phenylacetyl-CoA, the length of the rigid chains is 5 or 9 carbon atoms (Fig. 5). In either orientation (A or B), the size of the acyl-moiety is outside the critical limits required by AT and, therefore, they cannot be used as substrates.

ii. Substrates are Molecules Included in the A_2 Subgroup

The use of compounds in which the aromatic ring of PAA had been replaced by a different one (Fig. 6) led to the production of different penicillins, although in most cases at a lower rate. The highest quantities of penicillins were obtained when 2 or 3-thiopheneacetyl-CoA or 2 or 3-furoylacetyl-CoA was used as side-chain precursor whereas with cyclic aliphatic-CoA derivatives the amounts obtained were much lower[84]. These data indicate that the size of the ring as well as the existence of a rigid planar structure on the substrate molecule seem to be important for their utilization by AT.

iii. Substrates are Molecules Included in the B Group

Molecules, in which the benzene ring remained constant but with shorter or longer acyl-chains, (benzoyl-CoA, phenylpropionyl-CoA, phenylbutyryl-CoA, phenylvaleryl-CoA, phenylhexanoyl-CoA, phenylheptanoyl-CoA or phenyloctanoyl-CoA) were not recognized as substrates by AT[61]. Phenoxyacetyl-CoA, the side chain precursor of penicillin V (phenoxymethylpenicillin), was efficiently converted into this penicillin, reinforcing the hypothesis that AT would be the only enzyme which catalyzes the last biosynthetic step of all the hydrophobic penicillins obtained in industrial fermentations. The presence of an oxygen atom between the aromatic ring and the acetyl-CoA moiety is the sole modification on the acyl-chain accepted by the enzyme since replacement of the oxygen atom of phenoxyacetic acid (POA) by a sulfur atom (thiophenoxyacetyl-CoA), is sufficient to handicap this compound for use as a substrate[61].

A special group of substrates are those included in the B_2 subgroup; which correspond to molecules with substitutions on the α-carbon atom of phenylacetyl-CoA (Fig. 7). Using these compounds, penicillin synthesis was achieved when hydroxy, methyl or ethyl groups replaced a hydrogen atom, whereas other substitutions (amino, nitro or halogen atoms) were not tolerated[84].

Fig. 7. Structure of some phenylacetyl-CoA variants with modification on the α-carbon atom.

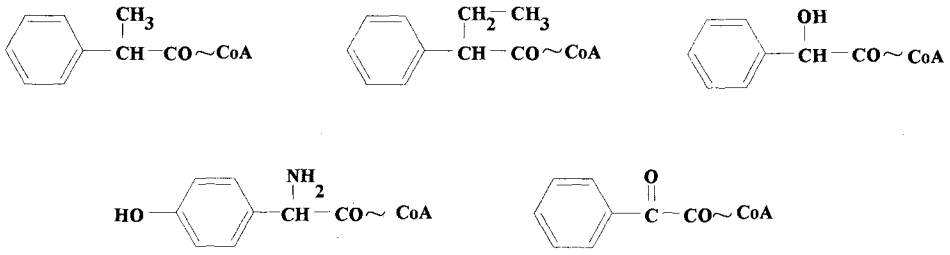
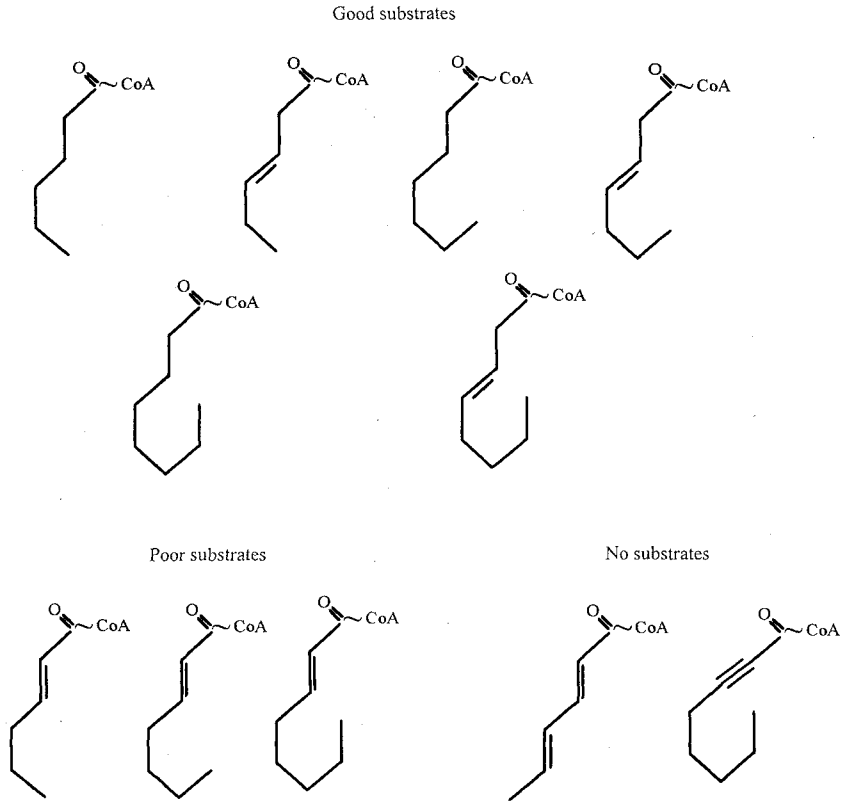


Fig. 8. Hypothetical conformations that could be adopted by aliphatic-CoA molecules and their influence on the recognition as substrates by AT.



iv. Substrates are Molecules Belonging to the C Group of Compounds

In vitro synthesis of benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V) was performed by direct acylation of 6-APA with (*S*-phenylacetyl)- and (*S*-phenoxyacetyl)-glutathione[82] and in both cases antibiotic synthesis was enhanced by CoA. In similar experiments, penicillins F, DF and K were synthesized when (*S*-3-hexenoyl)-, (*S*-hexanoyl)- or (*S*-octanoyl)-glutathione were incubated *in vitro* with 6-APA and AT. However, in these cases, CoA was an absolute requirement for the enzymatic production of these penicillins. Furthermore, benzylpenicillin was also obtained when the glutathione derivative (*S*-phenylacetyl)-cysteinyl-glycine was used as substrate, indicating that: (i) CoA could be replaced by other molecules, and (ii) some intermediates of the γ-glutamyl cycle could be directly involved in the biosynthesis of penicillins having molecules that cannot be synthesized by the producer organism as side-chains.

Therefore, it can be inferred that although the carbon length of the acyl moiety in the molecule of substrate is important for AT recognition, mobility at C_1, free rotation at C_2 and the volume of the side-chain precursor are also important characteristics determining which molecules can or cannot be efficiently incorporated into penicillins[12,61,84]. Thus, the utilization of very different molecules (PA-CoA, POA-CoA, 2- or 3-thiopheneacetyl-

Table 2. Comparative study of different acyl-CoA synthetases.

Enzyme (microbial source)	Mr	Optimum pH	Optimum T°	*Km* (substrate)	Reference
Acyl-CoA synthetase (*Pseudomonas fragi*)	60 kDa	7.5	37°C	2 mM (palmitate)	BOEHRINGER MANNHEIM, SA
Acyl-CoA synthetase (*Pseudomonas putida U*)	67 kDa	7.0	40°C	4 mM (acetate)	8
Acetyl-CoA synthetase (*Penicillium chrysogenum*)	140 kDa (dimer, α_2)	8.0	37°C	6.8 mM (acetate)	46
Acetyl-CoA synthetase (*Aspergillus nidulans*)	140 kDa (dimer, α_2)	8.0	37°C	5 mM (acetate)	46
Phenylacetyl-CoA ligase (*Pseudomonas putida U*)	48 kDa	8.2	30°C	16.6 mM (phenylacetate)	82

CoA, 2- or 3-furoylacetyl-CoA, hexanoyl-CoA, 3-hexenoyl-CoA, heptanoyl-CoA or octanoyl-CoA) as substrates by a single enzyme could be explained by taking into account that the aliphatic-CoA derivatives could acquire very similar steric conformations during catalysis (see Fig. 8). The lack of conversion observed when double or triple bonds are present at C_2 (which hinders the free rotation of this linkage and therefore avoids the steric adaptability of such molecules at the active site) further supports this assumption.

3. 6-Aminopenicillanic Acid is Replaced by other Molecules Containing the β-Lactam Ring

When 6-APA was replaced by 7-aminocephalosporanic acid (7-ACA) or 7-aminodeacetoxycephalosporanic acid (7-ADCA), no cephalosporin-like products were obtained[48,49,61]. Moreover, 3-aminomonobactamic acid could not be acylated either, suggesting that the thiazolidine ring is needed for catalysis. However, this cannot be the only requirement since 6-methyl- and 6-methoxy-6-APA were not acylated by AT even though the β-lactam and the thiazolidine rings were present (O. FERRERO and J. M. LUENGO, unpublished results).

B. Studies on Acyl-CoA Activating Enzymes

Once the substrate specificity of AT had been established, the study of some enzymatic systems that catalyze the activation of several acyl-acids to their CoA derivatives was approached. The aims of this second approach were dual. The first was to facilitate the synthesis of the CoA derivatives of many compounds that could require the protection of certain group(s) that hinder their chemical synthesis. The second was to study the physicochemical parameters, catalytic constants and optimal conditions for coupling these enzymes and AT *in vitro*.

Initial attempts were aimed at purifying of the specific enzyme (PCL) responsible for the activation of PAA to PA-CoA in *P. chrysogenum*. Unfortunately, after extensive research this study was abandoned since this enzyme could not be assayed by using previously reported procedures[52~53]. This problem was finally solved by using a different research strategy. It was asssumed that different species are able to employ analogous enzymes with different metabolic objectives[89~92]. Accordingly, several acyl-CoA ligases of different microbial origins were purified (see Table 2) and in each case the substrate specificity and the requirement for *in vitro* coupling with AT were established[84,85,90~94]. From among all the proteins studied, five enzymes were selected: phenylacetyl-CoA ligase and acetyl-CoA synthetase from *Pseudomonas putida* U; long-chain fatty acid-CoA ligase from *Pseudomonas fragi* and acetyl-CoA synthetases from *P. chrysogenum* and from *A. nidulans*. All these enzymes showed optimum assay conditions and physicochemical properties similar to those reported for AT, suggesting that they can be coupled *in vitro* and thus reproduce the last biosynthetic step leading to the enzymatic synthesis of hydrophobic penicillins. The data obtained in these experiments were the following:

1. Enzymatic Synthesis of Penicillins Using PCL from *P. putida* and AT from *P. chrysogenum* as the Coupled System

To study the *in vitro* synthesis of different penicillins by coupling PCL from *P. putida*[89] and AT from *P. chrysogenum*[61], these two enzymes were incubated with several molecules that could be used as penicillin side-chain precursors. Five groups of substrates were selected: (i) aliphatic acids (linear or branched, saturated or unsaturated); (ii) structural analogues of PAA with substitutions on the aromatic ring; (iii) PAA-analogues

with substitutions at the α-position; (iv) PAA-analogues containing a longer acyl-chain joined to the aromatic ring, and (v) compounds in which the PAA ring had been replaced by a different one. Evidence of penicillin formation was based on bioactivity towards *M. luteus*[40] as well as by analyzing the different antibiotics by HPLC[48~51]. With this system, sixty different penicillins were synthesized, including: natural penicillins (F, DF, K and others), some semisynthetic compounds (G, V, X and others) and also some penicillins, such as ticarcillin, that until now have only been obtained by chemical synthesis[84,85,89,93,94].

2. Enzymatic Synthesis of Penicillins Using Acyl-CoA Synthetase from *P. putida* (ACoAS) and AT from *P. chrysogenum* as the Coupled System

ACoAS was purified by growing *P. putida* U in a chemically defined medium containing octanoic acid as the sole carbon source. This enzyme is different from the PCL induced in this strain when grown in the same medium but replacing octanoic acid by PAA[92]. On using ACoAS-AT as the coupled system, penicillins were only synthesized when PAA, phenoxyacetic acid (POA) or aliphatic molecules with a carbon length between C_6-C_8 atoms were employed as penicillin side-chain precursors[92].

3. Enzymatic Synthesis of Penicillins Using Long-chain Fatty Acid-CoA Ligase (LFCoA-L) from the *Pseudomonas fragi*-AT Coupled System

Incubation of long-chain fatty acid-CoA ligase (AMP-forming) (LFCoA-L) from *P. fragi* with AT from *P. chrysogenum* in the presence of ATP, Mg^{2+}, CoA, DTT and 6-APA generated penicillins when hexanoic, *trans*-3-hexenoic, heptanoic, octanoic and *trans*-3-octenoic acids were used as substrates. However, no penicillins were synthesized when aromatic compounds (phenylacetic, phenoxyacetic, 2-furoylacetic, 3-furoylacetic, 2-thiopheneacetic and 3-thipheneacetic acids), which are usually good substrates for AT[49], were tested. Analysis of the quantity of penicillins produced indicated that the enzyme recognized those with a longer carbon chain (C_8) as better substrates[92].

4. Enzymatic Synthesis of Penicillins Using either Acetyl-CoA Synthetase (ACS) from *Penicillium chrysogenum* or *Aspergillus nidulans* and AT from *P. chrysogenum*: Role of ACS in the Transition of Primary to Secondary Metabolism

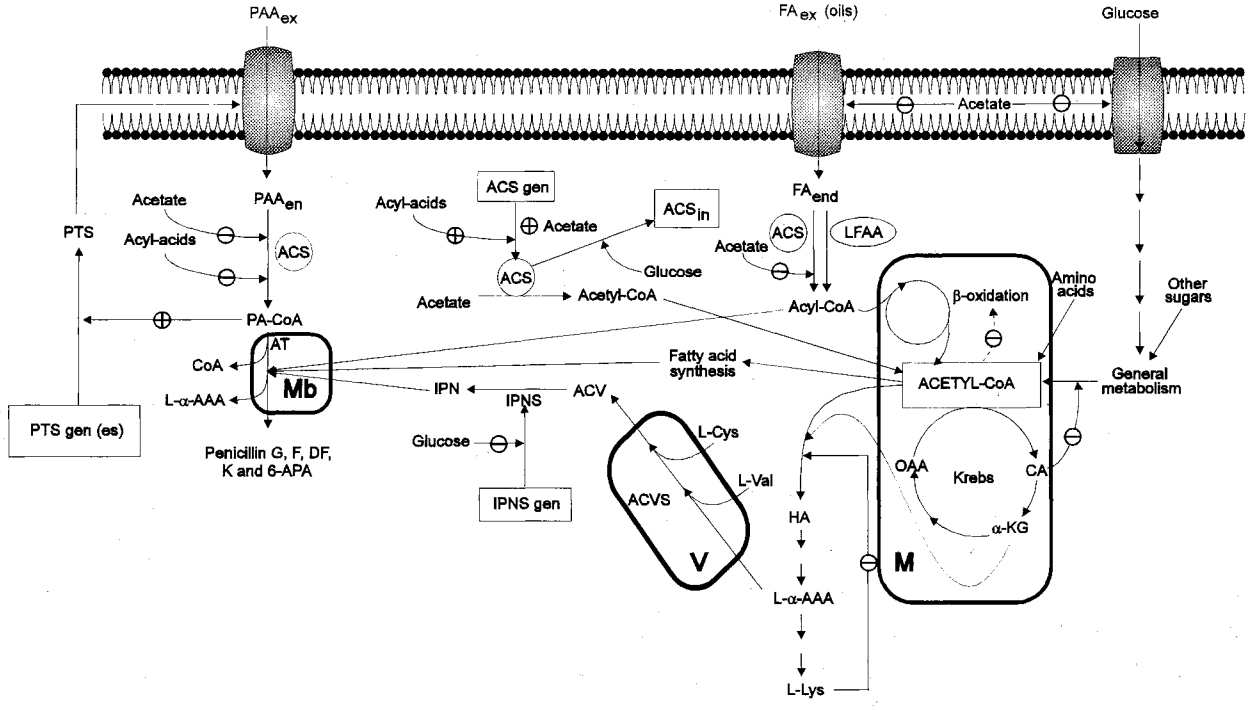
The synthesis of different penicillins when ACS and AT from *P. chrysogenum* and *A. nidulans* were coupled *in vitro* indicates that ACS could be the enzyme (or one of the enzymes) actually responsible for the activation, to their CoA derivatives, of the different side-chain precursors required by these two fungi for the production of such antibiotics *in vivo*. The direct involvement of ACS in the biosynthesis of penicillin G is supported by the fact that formation of this antibiotic was observed when the ACS-AT coupled system was incubated *in vitro* with PAA, CoA, ATP, Mg^{2+}, 6-APA[51]. However, penicillin G was not synthesized if acetate was supplied to the reaction mixture. In this reaction, mainly acetyl-CoA (but no or very little phenylacetyl-CoA) was formed and since acetyl-CoA is not a substrate of AT[49], no penicillin could be produced. By contrast, synthesis of penicillin G was detected when the concentration of acetate in the reaction was reduced[51]. This result is consistent with the absence of penicillin formation when acetate is catabolized as the carbon source. Similar data were obtained when the ACS-AT system was incubated with 3-hexenoic acid and acetate, strongly supporting the hypothesis that, *in vivo*, ACS could also catalyzes the activation (to their CoA derivatives) of other molecules used as penicillin-side chain precursors[51]. However, this situation (an excess of acetate or other acyl-acids with respect to PAA) would be a rare event in industrial fermentations of penicillin G, since continuous addition of PAA to the fermenters ensures the presence of high quantities of this compound, compensating for the lower affinity of ACS for phenylacetic acid.

The high quantity of penicillin F obtained when 3-hexenoic acid was tested as a substrate[51] indicates that *in vivo* this enzymatic system (ACS-AT) could probably produce higher amounts of this penicillin if the appropriate side chain precursors were present in the raw materials routinely used in industrial fermentations. This high capacity to synthesize penicillin F explains why this antibiotic is the only penicillin produced in the absence of PAA[51] and why it was the first penicillin to be isolated from the cultures of *P. chrysogenum*[7]. Moreover, penicillin F was not accumulated when PAA was added to the chemically defined medium, suggesting that 3-hexenoyl-CoA is not synthesized in the presence of phenylacetic acid.

In view of the involvement of ACS in the synthesis of substrates that can be used as side chain precursors of hydrophobic penicillins by AT, it could be speculated that in these fungi (*P. chrysogenum* and *A. nidulans*), the level of acetyl-CoA might be the metabolic signal that indicates the presence or not of rapidly metabolizable

substrates (which ensure the formation of Krebs cycle intermediates, and therefore a source of energy). If the acetyl-CoA pool (or the acetyl-CoA/CoA ratio) is high, the energy situation in the cells is favourable and we propose that this situation would correspond to primary metabolism. However, when a decrease in the acetyl-CoA pool occurs (*i.e.*: the acetyl-CoA/CoA ratio is low), this indicates that alternative sources of energy should be employed (secondary metabolism). Under these conditions, if acyl-acids exist intra- or extracellularly, ACS (or a similar enzyme) would synthesize acyl-CoA derivatives that could be used as penicillin side-chain precursors. The formation or not of benzylpenicillin would depend directly on the rate of phenylacetyl-CoA synthesis. Thus, if ACS recognizes other substrates better than PAA, the intracellular pool of PA-CoA will be low, the PATS will not be efficiently induced[47,51] and therefore no, or only a low, production of benzylpenicillin would be expected. This interpretation is consistent with the well known fact that natural penicillins are produced in industrial fermentation broths containing oils despite the presence of PAA (the side chain precursor of penicillin G)[7,51]. Under these culture conditions, the proteins, amino acids and other molecules contained in the raw materials ensure the formation of Krebs cycle intermediates, with the consequent excess of acyl-CoA derivatives (directly generated by fatty acid activation) and also an excess of acetyl-CoA (produced through fatty acid β-oxidation or through the glycolytic pathway) which are not immediately required to attend to cellular growth. In this metabolic situation, (i) some of these compounds (R-CoA, R ranges between C_6 to C_8 carbon atoms) will be recognized as substrates by AT, allowing the direct synthesis of natural penicillins (F, DF, K)[7,51] and (ii) the excess of acetyl-CoA could be efficiently used for fatty acid synthesis, thus generating the acyl-CoA derivatives required for the biosynthesis of these natural penicillins. However, we have shown[51] that this metabolic situation cannot be achieved until acetate has been exhausted since: (a) oil and glucose are not catabolized in the presence of acetate and (b) the acetyl-CoA synthesized by ACS must essentially be used to attend to the energy requirements of the growing cells. Study of the mechanism responsible for carbon catabolite regulation of PAA uptake in *A. nidulans* (FERNÁNDEZ-CAÑÓN and LUENGO, manuscript in preparation) highlights the regulatory role attributed to the acetyl-CoA molecule. We showed that PAA uptake is regulated in this

Fig. 9. Role of ACS and acetate on the biosynthesis of hydrophobic penicillins.



Integrative metabolic model. PAA, phenylacetic acid; PATS, phenylacetic acid transport system; ACS, acetyl-CoA synthetase; ACSin, ACS inactivated (by carbon catabolite regulation); FA, fatty acid; LFAA, long-fatty acid; PCL, phenylacetyl-CoA ligase; IPN, isopenicillin N; IPNS, isopenicillin N synthase; ACV, α-aminoadipyl-L-cysteinyl-D-valine; ACVS, ACV synthetase; CA, citric acid; α-KG, α-ketoglutaric acid; L-α-AAA, L-α-aminoadipic acid; en, endogenous; ex, exogenous. Boxes including AT and ACVS represent different subcellular organelles (Mb, microbody; V, vacuole and M, mitochondria, see text).

fungus by an unknown mechanism that is not mediated by the product of the *cre*A gene[95] since this uptake system is strongly repressed by glucose and glycerol in the *cre*A^{d} 30 mutant. Furthermore, when PAA uptake was studied in the mutant *fac*A 303, which lacks acetyl-CoA synthetase and is therefore handicapped in obtaining acetyl-CoA from acetate[96], we observed that when the fungus was grown in minimal medium containing PAA + acetate, PAA uptake was not affected whereas it was drastically repressed when the medium contained PAA + other carbon sources (glucose, glycerol, hexanoate) which can lead to acetyl-CoA by different pathways. In the light of the foregoing, we suggest that the acetyl-CoA pool could finely regulate the transition of primary to secondary metabolism in *P. chrysogenum* and in *A. nidulans*. A schematic representation of all these interpretations and an integrative model aimed at explaining the metabolic regulation of penicillin biosynthesis in these two fungi is summarized in Fig. 9.

IX. Concluding Remarks and Future Outlook

In view of the results published until now, it seems evident that AT is the only enzyme able to catalyze the last step of penicillin biosynthesis and that its substrate specificity is broad enough to produce more penicillins than those occurring naturally in industrial broths. Moreover, it has also been seen that several acyl-CoA ligases, obtained from different microbes (non-phylogenetically related) and with different sizes, characteristics and substrate specificities can be used in coupled systems with AT to enzymatically synthesize many hydrophobic penicillins, the only limitation being the substrate specificity of AT. The ability of several acyl-CoA ligases to activate penicillin side-chain precursors could be the reason why mutants of *P. chrysogenum* lacking this enzymatic activity (which should only produce IPN and 6-APA) have never been described.

Of special relevance are the results obtained with phenylacetyl-CoA ligase (PCL) from *P. putida*. This enzyme activates PAA to PA-CoA at a rate more than 500-fold higher that other acyl-CoA-activating enzymes. Furthermore, the PCL-AT coupled system is the most effective one found since more than 90% of the 6-APA present in the assay is converted into penicillin G[93]. The gene encoding PCL in *P. putida* has been recently cloned, sequenced and expressed in *E. coli* (LUENGO *et al.*, manuscript in preparation).

Taking the above considerations into account, it does not escape us that several future lines of research could be followed to obtain either overproducer mutants of *P. chrysogenum* or *A. nidulans* or to improve the titers of penicillins produced *in vitro*. Some of these could be the following:

a) Cloning of the PA-CoA ligase gene of *P. putida* in microbes which produce penicillin G. Expression of the PCL gene in *P. chrysogenum* or in *A. nidulans* could contribute to increasing the titers of penicillin G reached by these species if, as expected, PCL is a limiting step in the benzylpenicillin biosynthetic pathway. Furthermore, the fact that PCL from *P. putida* is quite different from the ACSs purified from these two fungi (regarding its nucleotide sequence, molecular size and specificity for substrates)[51,89] would suggest that the enzymes are under the control of different regulatory signals.

b) Isolation by reverse genetic techniques of different strains containing genes encoding acyl-CoA synthetases with a required substrate specificity. These mutants could be specifically used for the production of a particular type of penicillin (G, V, F, DF, K, *etc.*) or for a very close group of penicillins (aliphatic, aromatic, *etc.*).

c) Isolation of mutants containing genes (or proteins) insensitive to the regulatory signals that control their expression in the wild type strains. We speculate that manipulation of the genes coding for these enzymes could lead to "mutated" proteins providing new or modified β-lactam antibiotics.

d) Improvement of the physicochemical conditions or energy requirement needed for more efficient synthesis of the desired penicillin.

e) Study of the immobilization of PCL (or a different ligase) with AT in order to reproduce the synthesis of these β-lactam antibiotics from their elemental precursors.

It is therefore evident that research into penicillins, the first family of antibiotics discovered, continues to be an exciting and promising field which in the near future should offer fruitful results.

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