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Structure, Processing and Catalytic Action of Penicillin Acylase

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Introduction

Penicillin acylase enzymes (EC 3.5.1.11) are of considerable industrial importance, primarily in the production of 6-aminopenicillanic acid (6-APA) as an intermediate in the manufacture of semi-synthetic penicillins. Much research has been aimed at improving and extending the exploitation of this group of enzymes, and in the past decade useful progress has been made towards an understanding of the structure of penicillin acylase genes, the unusual mechanisms of processing of precursor proteins and some aspects of the structure and mode of action of the mature enzyme. The literature on applications of penicillin acylases, which is especially large in relation to immobilized enzyme preparations, is touched on briefly.

Sources of penicillin acylases

Penicillin acylases have often been isolated by means of screening naturally occurring candidate strains. Some mutant enzymes are discussed under 'Gene structure and expression' but serious application of the techniques of protein engineering awaits a high-resolution structure of the protein and details of its interactions with substrates and products.

DETECTION IN SOLUTION

The chromogenic substrate 6-nitro-3-phenylacetamido-benzoic acid (NIPAB), giving a product with a molar absorbance of 9100 M^{-1} cm^{-1},

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Abbreviations: 7-ACA, 7-aminocephalosporanic acid; 6-APA, 6-aminopenicillanic acid; CephC, cephalosporin C; NIPAB, 6-nitro-3-phenylacetamido-benzoic acid, *N*-(3-carboxy-4-nitrophenyl)-phenylacetamide; PenG, benzylpenicillin (penicillin G); PenV, phenoxymethylpenicillin (penicillin V); PMSF, phenylmethanesulphonyl fluoride.

provides a moderately sensitive means of measurement of penicillin acylase activity (Kutzbach and Rauenbusch, 1974). A colorimetric method using phenylacetylaminobenzoic acid as a substrate (Szewczuk, Siewinski and Slowinska, 1980) and a method based on thymol red (Wu et al., 1988) offer greater sensitivity by factors of four and three, respectively. The fluorescent product of reaction of fluorescamine with amino groups (Veronese et al., 1981) provides a very sensitive measure of activity. Fluorogenic substrates (Scheper, Weiss and Schugerl, 1988) combine sensitivity and convenience, but those available are of limited solubility in aqueous solvents. Finally, an enzyme-linked immunosorbent assay has been described for penicillin acylase (Prusak, Wieczorek and Szewczuk, 1987).

SCREENING

Table 1 shows a selection of methods and some further, older methods are given by Meevootisom et al. (1983) and by Vandamme and Voets (1974). Penicillin acylases are often expressed at low levels, conferring little resistance against β-lactam antibiotics and making chemical detection difficult. Thus, a method based on the biuret reaction (Baker, 1983b) was too insensitive for use in screening. Also, the presence of the enzyme is readily masked by β-lactamase (EC 3.5.2.6) activity (Cole, Savidge and Vanderhaeghe, 1975). Some microbiological methods have made use of marker organisms which are resistant to the desired β-lactam substrate but sensitive to the product, either 6-APA or 7-aminocephalosporanic acid (7-ACA). Alternatively, the ability to grow on the amide corresponding to the desired penicillin side-chain has been used as the basis of selection.

Table 1. Screening methods

Method (substrate)	References
Microbiological	
Growth on amide (PenV)	Vojtisek et al. (1987); Slezak et al. (1987)
Product-sensitive strain (PenG,V)	Meevootisom et al. (1983)
Product-sensitive strain (CephC)	Boehringer-Mannheim GmbH (1988)
Chemical	
6-APA/fluorescamine (PenG)	Baker (1983a)
6-APA/fluorescamine (PenG,V, ampicillin)	Baker (1984)
Separation of dansyl-derivatives (ampicillin, cephalexin)	Chen (1986)
NIPAB test paper (PenG)	Zhang et al. (1988)

DISTRIBUTION AND FUNCTIONAL SIGNIFICANCE

Penicillin acylases are widely, though sparsely, distributed among micro-organisms, including bacteria, actinomycetes, yeasts and fungi. The physiological significance is uncertain and may not be the same in all organisms: classification by substrate specificity will not necessarily produce the same result as classification by molecular structure.

Classification by substrate specificity

In the absence of structural information, attempts have been made to classify penicillin acylases on the basis of their microbial origins and on their preferred substrate. There are well-documented differences in specificity for the penicillin side-chain, but the distinction between penicillin and cephalosporin acylases is less clear. Thus, the published names, followed in this review, do not necessarily imply that the enzyme is exclusively active towards only one class of β-lactams.

Early work, reviewed by Hamilton-Miller (1966), established that, with a significant number of exceptions, fungal enzymes hydrolysed the side-chain of phenoxymethylpenicillin (penicillin V; PenV) in preference to that of benzylpenicillin (penicillin G; PenG), while bacterial enzymes showed the converse preference. Some authors have designated PenV acylases and PenG acylases as types I and II, respectively, although Abbott (1976) reverses this order. Further enzyme types have been proposed, including ampicillin acylases which have been designated type III. Vandamme and Voets (1974) have reviewed the occurrence of these and some other, possibly different, enzyme types. The frequency of occurrence of promising levels of PenV acylase in more than 2000 isolates of soil bacteria was described by Lowe, Romancik and Elander (1981).

Substrate specificity studies

An early study of the specificity of *Escherichia coli* PenG acylase was carried out using an unpurified, cell-bound enzyme preparation (Cole, 1969a). It is probable that the presence of cell membranes impeded the diffusion of reactants so that the apparent K_m for PenG (Cole, 1969a) was greater than the best values found for purified, soluble preparations [diffusion effects may also account for the very high K_m and product K_i values determined by Schomer, Segner and Wagner (1984) for penicillin acylase in whole *E. coli* cells]. In order of the rate of deacylation, the most susceptible penicillin substrates were *p*-hydroxybenzyl-, DL-α-hydroxybenzyl-, 2-furylmethyl-, 2-thienylmethyl-, D-α-aminobenzyl-, *n*-propoxymethyl- and isobutoxymethyl-penicillin. Phenylpenicillin and DL-α-carboxybenzylpenicillin were not substrates and PenV was a poor substrate. Amides and esters of penicillins, and cephalosporins with a thienylmethyl side-chain, were also substrates (Cole, 1969a). Compounds other than penicillins, including phenylacetylglycine and amides such as phenylacetamide, were substrates (Cole, 1969b). Essentially the same specificity was found in the direction of synthesis of penicillins and other acylamino compounds, and the reaction was most effective when an amide or *N*-acylglycine was the acyl group donor in a transacylation reaction (Cole, 1969c).

Physiological function

Penicillin acylase is usually present in organisms that synthesize penicillin, but the physiological role of the enzyme is uncertain (Vandamme, 1977). The

enzyme probably only accounts for a small degree of resistance to β-lactam antibiotics compared with the major role of the β-lactamases, but it may be significant in some organisms. For example, Pavlovich, Shimaniak and Mishan'kin (1983) reported that highly resistant strains of *Francisella tularensis* all possessed both β-lactamase and penicillin acylase activity. Recently, an increase in PenG and PenV acylase activity has been observed during the autolysis of filamentous fungi (Alfonso, Cribeiro and Reyes, 1989).

Table 2. Purified soluble penicillin acylase preparations

Source		References
Bacteria (PenG acylase)		
Arthrobacter viscosus	8895GU	Ohashi *et al.* (1988)
Bacillus megaterium		Chiang and Bennett (1967)
		Lim and Byun (1981)
Escherichia coli	NCIB 8743A	Balasingham *et al.* (1972)
	ATCC 11105	Kutzbach and Rauenbusch (1974)
	ATCC 14945,	
	NCIB 8743A	Savidge and Cole (1975)
	SV	Veronese *et al.* (1981)
	–	Vojtisek *et al.* (1980)
	NCIM 2500	Mahajan and Borkar (1982, 1984)
	–	Hindustan Antibiotics Ltd (1984a,b)
	–	Kasche, Haufler and Zollner (1984)
	PCM 271	Szewezuk *et al.* (1984)
	AS 1-76	Jiao, Zhang and Wang (1986)
	–	Antibioticos S.A. (1985)
	–	Hustedt and Papamichael (1988)
Proteus rettgeri		Robak and Szewczuk (1981)
Kluyvera citrophila	KY7844	Shimizu, Okachi and Nara (1975)
	ATCC 21285	Barbero *et al.* (1986)
	–	Antibioticos S.A. (1985)
Bacteria (PenV acylase)		
Bacillus megaterium	14945	Meevootisom and Saunders (1987)
Bacillus sphaericus		Olsson *et al.* (1985)
B. subtilis	pOH38	Gatenbeck *et al.* (1986)
Erwinia aroideae		Vandamme and Voets (1975)
Bacteria (ampicillin acylase)		
Pseudomonas melanogenum	–	Hur *et al.* (1987)
Bacteria (GL-7ACA acylase)		
Pseudomonas sp.	SE83 (acyII)	Matsuda *et al.* (1987)
		Asahi Chemical Industry Co.Ltd (1987a,b)
	GK16	Matsuda and Komatsu (1985)
Bacteria (Cephalexin acylase)		
Xanthomonas sp.	–	Petrova, Penzikova and Levitov (1984)
Actinomycetes (PenV acylase)		
Streptoverticillium	No. 62	Borisov *et al.* (1984)
Fungi (PenV acylase)		
Bovista plumbea	–	Schneider and Roehr (1976)
Fusarium semitectum	–	Waldschmidt-Leitz and Bretzel (1964)
	–	Baumann, Brunner and Roehr (1971)
	BC805	Vanderhaeghe (1975)
F. avenaceum	–	Vanderhaeghe (1975)
F. oxysporum	–	Lowe, Romancik and Elander (1986)
Penicillium chrysogenum	–	Vanderhaeghe (1975)
Fungi (CephC acylase)		
Paecilomyces sp.	C-2106	Kawate *et al.* (1987)

SOURCES OF PURIFIED ENZYMES

Purified preparations have been obtained from a relatively small proportion of the organisms identified as producing a penicillin acylase. Some sources of enzymes relevant to antibiotic production have been reviewed by Sudhakaran and Borkar (1985a,b). Studies using a number of cloned gene products are discussed in other sections.

Soluble enzyme preparations

Following the partial purification of the enzyme from several microbial sources, the enzyme from *E. coli* ATCC 11105 was the first to be purified to homogeneity and to be crystallized. *Table 2* gives a summary of sources of purified enzyme preparations, including some of partial or uncertain purity. Methods of isolation and purification are discussed in practical detail by Savidge and Cole (1975) and by Vanderhaeghe (1975). Affinity purification methods relevant to penicillin acylase have been discussed by Mahajan and Borkar (1984) and a recent affinity purification method is given by Karyekar and Hegde (1989). Some of the physicochemical properties of purified enzyme preparations have been reviewed by Mahajan (1984).

   In assessing the purity of preparations it is important to keep in mind that some organisms may produce more than one form of penicillin acylase (Kutzbach and Rauenbusch, 1974; Kasche, Haufler and Zollner, 1984; Kasche *et al.*, 1987), more than one cephalosporin acylase (Oreshina *et al.*, 1984; Matsuda *et al.*, 1987) or a penicillin acylase together with a related enzyme activity, such as aminoacylase (Borisov *et al.*, 1984). Szewczuk *et al.* (1984) found that two electrophoretically and immunochemically distinct forms of PenG acylase were produced by *E. coli* PCM 271 cells in proportions dependent on the growth conditions. In terms of immunological cross-reactivity, *E. coli* ATCC 11105 cells contained both forms while only one form was produced in cells of strains ATCC 9636 and ATCC 9637 (Szewczuk, Kurowska and Wieczorek, 1987).

Reactions catalysed and their applications

REACTION EQUILIBRIUM

The equilibrium constant for hydrolysis of PenG to 6-APA and phenylacetic acid is strongly pH-dependent, favouring the forward reaction at alkaline pH values (Svedas, Margolin and Berezin, 1980; Konecny, 1983). Detailed equilibrium measurements and microcalorimetry (Tewari and Goldberg, 1988) yielded the following parameters for the reaction:

$$PenG^-(aq.) + H_2O(l) = \text{phenylacetic acid}^-(aq.) + 6\text{-APA}^-(aq.) + H^+(aq.)$$
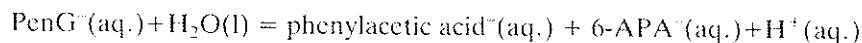
$$K = (7 \cdot 35 \pm 1 \cdot 5) \times 10^{-8} \text{ mol kg}^{-1};$$

$$\triangle G^0 = 40 \cdot 7 \pm 0 \cdot 5 \text{ kJ mol}^{-1};$$

$$\triangle H^0 = 29 \cdot 7 \pm 0 \cdot 6 \text{ kJ mol}^{-1};$$

$$\triangle C_p^\circ = -240 \pm 50 \text{ J mol}^{-1} \text{ K}^{-1} \text{ at } 298 \cdot 15\text{K and}$$
at the thermochemical standard state.

Equilibrium constants have been reported, though in less detail, for other reactions, for example ampicillin hydrolysis in the pH range 4·5–5·5 (Margolin *et al.*, 1978).

APPLICATIONS

The hydrolysis reaction has been the more important industrially, but attempts have been made to develop the reverse (synthetic) direction, making use of *trans*-acylation reactions, for semi-synthesis of penicillins and cephalosporins. The ability of penicillin acylase to accept a rather wide range of amides and esters of a small number of acyl side-chains has also prompted a variety of other potential applications.

Immobilized enzymes

Abbott (1976) described the range of immobilized enzyme preparations then available, including fungal spores, bacterial cells trapped in polyacrylamide gel or in cellulose triacetate fibres, and enzymes chemically coupled to several different supports. Subsequently there have been many reports of immobilized enzyme preparations using liquid membranes, gels, foams, beads, granules, fibres, films, fabrics, porous glasses and microbial aggregates (Kennedy, Melo and Jumel, 1989). Improved stability during repeated cycles of use has been an important objective in the development of immobilized enzyme preparations and some of the principles of stabilization against thermal inactivation have been discussed by Klibanov (1983). An increase in apparent K_m is commonly observed when enzymes are immobilized and penicillin acylase is no exception (Carleysmith, Dunnill and Lilly, 1980). Margolin *et al.* (1985) assessed the effects of immobilizing penicillin acylase in a polyelectroyte complex formed by modified poly(N-ethyl-4-vinyl-pyridinium bromide) and an excess of poly(methylacrylic acid). The polymer was soluble at pH 6·1 and above, but precipitated at pH 5·7 and, since the phase change did not affect the number of cross-links between the polymer and the enzyme, it provided an internal control for determining the effects of immobilization. In terms of thermal inactivation, there was a marked stabilization of the enzyme in the solid phase but a decreased activity associated with a tenfold increase in K_m.

Production of 6-APA

The major use of penicillin acylase has been in the deacylation of PenG or PenV to obtain 6-APA, and aspects of this have been reviewed recently

(Shewale and Sivaraman, 1989). Product inhibition by phenylacetic acid remains a problem in limiting the rate of completion of the reaction.

Production of 7-ACA

An enzyme with good activity towards the α-aminoadipyl side-chain of cephalosporin C (CephC) has been elusive so that a route to 7-ACA has not been straightforward. For example, the recently sequenced *Pseudomonas* enzyme, described as active towards CephC, is only 5% as active with this substrate as with the best substrate found (Matsuda *et al.*, 1987). Other enzymes with a possibly useful activity towards CephC include a preparation from *Arthrobacter viscosus* which slowly released 7-ACA from CephC (Merck and Co. Inc., 1988) and a partially purified enzyme from mycelia of *Paecilomyces* sp. C-2106 (Kawate *et al.*, 1987).

Removal of protecting groups

The specificity of PenG acylases towards the phenylacetyl part of the substrate has suggested the use of the enzyme to remove an *N*-phenylacetyl protecting group under mild aqueous conditions. Some recent examples are: insulin (Wang *et al.*, 1986), aspartame (Fuganti and Grasselli, 1986), peptides (Waldmann, 1988a,b) and sugar derivatives (Waldmann, 1988a). Baldaro *et al.* (1988) proposed the phenylacetyloxymethylene group for protection of the carboxyl group of PenG during ring expansion reactions to obtain 7-ACA derivatives, penicillin acylase being used to remove both the protecting group and the phenylacetyl group at the 7-position.

Resolution of diastereoisomers

There is stereoselectivity in the reactions catalysed by PenG acylase both for substituents at the prochiral methylene group of phenylacetic acid and for the amine or alcohol moiety (Cole, 1969a,b). A high degree of selectivity is found towards derivatives of L-amino acids but the enantiomeric excess of products is often lower with other compounds (Rossi and Calcagni, 1985; Fuganti *et al.*, 1986, 1987, 1988).

Acylation reactions

The ability of penicillin acylase to catalyse *trans*-acylation reactions (Cole, 1969c) permits suitable esters to be used to donate a side-chain group, as illustrated by an early report of the synthesis of penicillins and cephalosporins, including the formation of ampicillin and amoxycillin by *trans*-acylation from esters of phenylglycine and *p*-hydroxyphenylglycine, respectively (Marconi *et al.*, 1975). The permitted variation of the side-chain structure is constrained by the enzyme specificity, but the relatively low specificity for the acceptor nucleophile allows synthesis of a variety of products. Compounds other than penicillins and cephalosporins which may be acylated include: monobactams (O'Sullivan and Aklonis, 1984), and a number of amino acid

esters, dipeptides and tripeptides with yields of 10–80% (Pessina *et al.*, 1988).

A complication in the exploitation of such reactions is the existence of a kinetically controlled maximum yield (Cole, 1969d; Svedas *et al.*, 1980; Kasche and Galunsky, 1982; Kasche, Haufler and Zollner, 1984; Kasche, Haufler and Riechmann, 1984; 1987; Kasche, 1986). This arises because of the competition between *trans*-acylation and hydrolysis, so that the amide product continues to accumulate only until the compound donating the acyl group approaches exhaustion.

Enzyme structure

Progress has been made in determining the amino-acid sequence and subunit structure of several related PenG acylases and cephalosporin acylases. One of the PenV acylases appears to be unrelated to the other enzymes. To date, no three-dimensional structure has been completed. Other proteins recognizing β-lactams (Coulson, 1985) are apparently unrelated to the penicillin acylases.

SUBUNIT COMPOSITION

Using gel filtration, Kutzbach and Rauenbusch (1974) found what is now recognizable as an anomalously low value (70 000 Da) for the molecular mass of the enzyme from *E. coli* ATCC 11105. The presence of two polypeptide chains (M_r = 20 500 and 71 000, respectively) in SDS-polyacrylamide gel electrophoresis, together with some evidence of interconversion of the two species, was therefore misinterpreted in terms of incomplete dissociation of the enzyme into subunits of the lower molecular mass. A similar erroneous conclusion was reached by Shimizu, Okachi and Nara (1975) for the enzyme from *Kluyvera citrophila*.

Subsequently, Böck *et al.* (1983a) showed that the periplasmic form of the *E. coli* enzyme was composed of two subunits with different molecular masses on SDS-polyacrylamide gel electrophoresis (20 500 and 69 000 Da, respectively). There was no evidence of interconversion and antisera raised against the isolated β-subunits did not cross-react with the α-subunit.

A similar subunit structure has been reported for the enzyme from *Proteus rettgeri* (Daumy, Danley and McColl, 1985) and similar structures may be inferred from sequencing of cephalosporin acylases, discussed below. However, the enzyme purified from *Bacillus sphaericus* and the enzyme obtained from heterologous expression of the gene in *E. coli* possessed a molecular mass suggesting a homotetramer with a subunit molecular mass of 35 000 Da (Olsson *et al.*, 1985).

Gene structure and expression

Genes of penicillin and cephalosporin acylases have been cloned from a number of species, including *E. coli* (Mayer, Collins and Wagner, 1979, 1980), *K. citrophila* (García and Buesa, 1986), *Bacillus megaterium* (McCullough, 1983), *Acetobacter turbidans* (Nam and Ryu, 1988) and other species

mentioned below. Partial or complete sequences of the protein-coding regions have been established for most of these and, in some cases, the 5'- and 3'-sequences flanking the open reading frame have been determined. Some aspects of the molecular biology of penicillin acylase have been reviewed recently (Francetic, Marjanovic and Glisin, 1988).

PROTEIN-CODING REGION

Table 3 shows a selection of complete and partial amino-acid sequences of penicillin acylases and cephalosporin acylases, aligned according to published sequence similarities, so that the sequence indices in *Table 3* differ from published sequence numbering according to the number of gaps (-) introduced. Both nucleotide sequencing and limited amino-acid sequencing (underlined residues) have played an important part in establishing the subunit structure of the *E. coli* PenG acylase and some other acylases.

There are relatively few differences between the complete sequence of the penicillin acylase from *E. coli* ATCC 11105 shown in *Table 3* (Schumacher *et al.*, 1986) and other published partial and complete sequences for the enzyme from the same strain (Brüning *et al.*, 1984; Bruns *et al.*, 1985; Oliver *et al.*, 1985; Valle *et al.*, 1986; Oh *et al.*, 1987). The penicillin acylase gene and its product from *E. coli* AS 1·76 has been reported to possess nucleotide and amino-acid sequences which are virtually identical to those of strain ATCC 11105 (Guo *et al.*, 1989). The genes from *E. coli* and *K. citrophila* are 80% homologous and there is a comparable (87%) degree of similarity in the coded amino-acid sequence (Barbero *et al.*, 1986). N-terminal amino-acid sequences of the mature enzyme from *A. viscosus* (PACAV) are also clearly homologous (Ohashi *et al.*, 1988). Two of the three genes for cephalosporin acylases isolated from *Pseudomonas* sp. show regions of sequence homology with those of *E. coli* and *K. citrophila*. The *acyII* gene of *Pseudomonas* sp. SE83 (Matsuda, Toma and Komatsu, 1987) and the partially sequenced gene from *Pseudomonas* sp. GK16 (Matsuda and Komatsu, 1985) show some regions of homology with each other and with the penicillin acylase (7B4CPS, ACYIIPS, ACYIPS) sequences. These fall into two groups: a region in the α-subunit (*Table 3*, positions 48–142) and a shorter region at the N-terminus of the β-chain. *Table 3* positions 305–326 were identified by Matsuda, Toma and Komatsu (1987) but the similarities appear to extend somewhat further. No basis has been found for alignment of the *Pseudomonas acyI* acylase with the other sequences, although N-terminal sequencing suggests that proteolytic cleavage occurs during processing to yield two subunits. However, in this case the proposed N-terminal subunit is the larger (Matsuda, Toma and Komatsu, 1987). The *B. sphaericus* PenV acylase (PACBS), which as noted above is apparently a homotetramer, shows no obvious relationship with the α- or β-subunits of the other acylases.

Mutagenesis of the protein-coding region

Advantage has been taken of the availability of cloned genes to produce and/or to characterize mutant enzymes with altered functional properties.

Table 3.   Partial alignment of amino-acid sequences

Enzyme	References
Penicillin G acylases	
E. coli ATCC 11105 (PACEC)	Schumacher et al. (1986)
K. citrophila (PACKC)	Barbeto et al. (1986)
A. viscosus (PACAV)	Ohashi et al. (1988)
Cephalosporin acylases	
Ps. sp. GK16 (7B4CPS)	Matsuda and Komatsu (1985)
Ps. sp. SE83 (ACYHPS)	Matsuda, Toma and Komatsu (1987)
Ps. sp. SE83 (ACYIPS)	Matsuda, Toma and Komatsu (1987)
Penicillin V acylase	
B. sphaericus (PACBS)	Olsson and Uhlén (1986)

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              1
PACEC    MKNRNRMIVN CVTASLMYYW SLPALA---- ---------- EQSSSEIKIV
PACKC    MKNRNRMIVN GIVTSLICCS SLSALA---- ---------- ASPPTEVKIV
PACAV    ---------- ---------- ---------- ---------- -DKNEGVKVV
7B4CPS   MLRVLHRAAS ALVMATVIGL APAVAFALAE PTSTPQAPIA AYKPRSNEIL
ACYIIPS  MTMAAKTDRE ALQAALPPLS GGLSIPGLSA P--------- ------VRVQ
ACYIPS   MNAPVPVPRV ADFTCEKKPA SGSRGMVVTN HPLASAAGAQ ILLAGGNAID
PACBS    MLGCSSLSIR TTDDKSLFAR TMDFTMEPDS KVIIVPRNYG IRLLEKENVV


              51
PACEC    RDEYGMPHIY ANDTWHLFYG YGYVVAQDRL FQMEMARRST QGTVAEVLGK
PACKC    RDEYGMPHIY ADDTYRLFYG YGYVVAQDRL FQMEMARRST QGTVSEVLGK
PACAV    RDRFGVPHLY AKNKKDLYEA YGYVMAKDRL FQ-------- ----------
7B4CPS   WDGYGVPHIY GVDAPSAFYG YGWAQARSQG DNILRLYGEA RGKGAEYWGP
ACYIIPS  RDGWGIPHIK ASGEADAYRA LGFVHAQDRL FQMELTRRKA LGRAAEWLGA
ACYIPS   AAVASLFALT VAEPMMVGIL GGGLSHIRLA DGRHVVIDNL STAPGKRATAE
PACBS    INNSYAFVGM GSTDITSFVL YDGVNEKGLM GAMLYYATFA TYADEPKKGT


              101
PACEC    DFVKFDKDIR RNYWPDAIRA QIAALSPEDM SILQGYADGM NAWIDKVNTN
PACKC    AFVSFDKDIR QNYWPDSIRA QIASLSAEDK SILQGYADGM NAWIDKVNAS
PACAV    ---------- ---------- ---------- ---------- ----------
7B4CPS   DYEQTTVWLL THGVPERAQQ WYAQQSPDFR ANLDAFAAGI NAYAQQNPDD
ACYIIPS  EAAEADILVR RLGMEKVCRR DFEALGAEAK DMLRAYVAGV NAFLASGAPL
ACYIPS   MYECLSDEIG KQRDTRDRQN VVGAKAVAVP GALKGWCEAL ARFGTLPLAE
PACBS    TGINPVYVIS QVLGNCVTVD DVIEKLTSYT LLNEANIILG FAPPLHYTFT


              151
PACEC    PETLLPKQFN TFGFTPKRWE PFDVAMIFVG TMANRFSDST SEIDNLALLT
PACKC    PDKLLPQQFS TFGFKPKHWE PFDVAMIFVG TMANRFSDST SEIDNLALLT
PACAV    ---------- ---------- ---------- ---------- ----------
7B4CPS   ISPDVRQVLP VSGADVVAHA HRLMNFLYVA SPGRTLGEGD PPDLADQG--
ACYIIPS  PIEYGLLGAE PEPWEPWHSI AVMRRLGLLM GSVWFKLWRM LALFVVGAAN
ACYIPS   VLQPAIGLAE RGFVVTPYLS NCITDNAGDL ARDPGLAAML LPGGKFLQFG
PACBS    DASGESIVIE PDKTGITIHR KTIGVHTNSF GYEWHQTNLR AYIGVTPNFF
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                201
PACEC           ALKDKYGVSQ GMAVFNQLKW LVNPSAFTTI AVQESNYPLK FNQQNSQTAA
PACKC           AVKDKYGNDE GMAVFNQLKW LVNPSAFTTI AARESSYPLK FDLQNTQTAA
PACAV           .... .....  .......... .......... .......... ......... 
7B4CPS          .... .....  .......... .......... .......... ......... 
ACYIIPS         ALKLRYDDGG QDLLCIFFGV EAERLEADLA ALRFAVDALL KAMGGDACDA
ACYIPS          MRLVQSDYAA SLKLIAAEGP DALYGGKLGE ALTDYMAANG GLIDQADLAN
PACBS           QDIMMGDLDL TFFGQGAGGL GLFGDFTPSA RFLRVAYWKK YTEKAKNETE


                251
PACEC           LL-PRYDLFA PMLDRFAKGA DGALLALTAG KNRETIAAQF AQGGANGLAG
PACKC           LLVPRYDQPA PMLDRPAKGT DGALLAVTAI KNRETIAAQF ANG-ANGLAG
PACAV           .......... .......... .......... .......... ....... ...
7B4CPS          .......... .......... .......... .......... ....... ...
ACYIIPS         AGGG------ .......... .......... .......... ....... ...
ACYIPS          YRIELREFIR GSYRGYEIIG FFPPSSSGVH ITQMLNILEG YDIGSLGFGS
PACBS           GVTNLFHILS SVNIFKGVVL TNEGKTDYTI YTSAMCAQSK NYYFKLYDRG


                301
PACEC           YPTTSNMWVI GKSKAQDAKA IMVNGPQFGW YAPAYTYGIG LHGAGYDVTG
PACKC           YPTTSNMWVI GKNKAQDAKA IMVNGPQFGW YAPAYTYGIG LHGAGYDVTG
PACAV           ---SNAAIV GSEKSATGNA LLFSGPQVSF VAPGFLYEVG LH
7B4CPS          --SNSWAV APGKTANGNA LLLQNPHLSW TTDYFTYYEA HLVTPDFEIY
ACYIIPS         ----SNNWAV APGRTATGRF ILAGDPHRVF EIPGMYAQHH LACDRFDMIG
ACYIPS          TPAVHLLAEA LKIAFADRAV ATADFAFVKV PVARLIDKAY ADERRALIEM
PACBS           FISAVSLMAE NLNSQDLITF EWDRKQDIKQ LNQVNVMS


                351
PACEC           NTPFAYFGLV FGHNGVISWG STAGFGDDVD IFAERLSAEK PGYYLHNGKW
PACKC           NTPFAYFGLV FGHNGTISWG STAGFGDDVD IFAEKLSAEK PGYYQHNGEW
PACAV           
7B4CPS          GATQIGLFVI RFAFNQRMGI TNTVNGMVGA TNYRLTLQDG GYLYDGQVEP
ACYIIPS         LTVPGVEGFP RFAHNGKVAY CVTHAFMDIH DLYLEQFAED GRTARFGNEF
ACYIPS          EQAKSWTAGL SGGESADTTH VTYADAMGNV VSATQTINGI FGACVQIPGT
PACBS           


                401
PACEC           VKMLSREETI TVKNGQAETF TVWRTVHGNI LQTDQTTQTA YAKSRAWDGK
PACKC           VKMLSRKETI AVKDGQFETF TVWRTLDGNV IKTDTRTQTA YAKARAWAGK
PACAV           
7B4CPS          FERFQASYRL RQADGTT
ACYIIPS         EPVAWRRDRI AVRGGADREF DIVETRHGPV IAGDPLEGAA LTLRSVQFAE
ACYIPS          GHIANNYMYN FDFHPGRALS IAPGKRVFTS MAFMMALKEG RIAFALGLPG
PACBS           
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                      451
PACEC      EVASLLAWTH QMKAKNWQEW TQQAAKQALT INWYYADVNG NIGYVHTGAY
PACKC      EVASLLAWTH QMKAKNWPEW TQQAAKQALT INWYYADVNG NIGYVHTGAY
PACAV
7B4CPS
ACYIIPS    TDLSFDCLTR MPGASTVAQL YDATRGWGLI DHNLVAGDVA GSIGHLVRAR
ACYIPS     ALRIFPSALQ AIVNLIDHRM SLQEAVEAPR VWTEGGVLEL EEAIPEAVAQ
PACBS


                      501
PACEC      PDRQSGHDPR LPVPGTGKWD WKGLLPFEHN PKVYNPQSGY IANWNNSPQK
PACKC      PDRQPGHDPR LPVPD-GKWD WKGLLSFDLN PKVYNPQSGY IANWNNSPQK
PACAV
7B4CPS
ACYIIPS    VPSRPRENGW LPVPGWSGEH EWRGWIPHEA MPRVIDPPGG LIVTANNRVV
ACYIPS     ALIARGHKVV RSPRVAGGMN AIAFNPDGTL TGAACWRADG TPVAISGGLA
PACBS


                      551
PACEC      DYPASDLFAF LWGGADRVTE IDRLLEQKPR LTADQAWDVI RQTSRQDLNL
PACKC      DYPASDLFAF LWGGADRVTE IDTILDKQPR FTADQAWDVI RQTSLRDL-L
PACAV
7B4CPS
ACYIIPS    ADDHPDYLCT DCHPPYRAER IMERLVASPA FAVDDAAAIH ADTLSPHVGL
ACYIPS     RAGARFTI
PACBS


                      601
PACEC      RLFLPTLQAA TSGLTQSDPR RQLVETLTRW DGINLLNDDG KTWQQPGSAI
PACKC      RLFLPALKDA TANLAENDPR RQLVDKLASW DGENLVNDDG KTYQQPGSAI
PACAV
7B4CPS
ACYIIPS    LRARLEALGI QGSLPAEELR QTLIAWDGRM DAGSQAASAY NAFRRALTRL
ACYIPS
PACBS


                      651
PACEC      LNVWLTSHLK RTVVAAVPHP FDKWYSASGY ETTQDGPTGS LNISVGAKIL
PACKC      LNAWLTSHLK RTVVAAVPAP FGKWYSASGY ETTQDGPTGS LNISVGAKIL
PACAV
7B4CPS
ACYIIPS    VTARSGLEQA IAHPFAAVPP GVSPQGQVWW AVPTLLRNDD AGHLKGWSWD
ACYIPS
PACBS
```

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                   701
PACEC              YEAVQGDKSP IPQAVDLFAG KPQQEVVLAA LEDTWETLSK RYGNNVSNWK

PACKC              YEALQGDKSP IPQAVDLFGG KPEQEVILAA LDDAWQTLSK RYGNDVTGWK

PACAV

7B4CPS

ACYIIPS            EALSEALSVA TQNLTGRGWG EEHRPRFTHP LSAQFPAWAA LLNPVSRPIG

ACYIPS

PACBS



                   751
PACEC              TPAMALTFRA NNFFGVPQAA AEETRHQAEY QNRGTENDMI VFSPTTSDRP

PACKC              TPAMALTFRA NNFFGVPQAA AKEARHQAEY QNRGTENDMI VFSPTSGNRP

PACAV

7B4CPS

ACYIIPS            GDGDTVLANG LVPSAGPEAT YGALSRYVFD VGNWDNSRWV VFHGASGHPA

ACYIPS

PACBS



                   801
PACEC              VLAWDVVAPG QSGFIAPDGT VDKHYEDQLK MYENFGRKSL WLTKQDVEAH

PACKC              VLAWDVVAPG QSGFIAPDGK ADKHYDDQLK MYESFGRKSL WLTPQDVDEH

PACAV

7B4CPS

ACYIIPS            SPHYADQNAP WSDCAMVPML YSWDRIAAEA VTSQELVPA

ACYIPS

PACBS



                   851
PACEC              KESQEVLHVQ R

PACKC              KESQEVLQVQ R

PACAV

7B4CPS

ACYIIPS

ACYIPS

PACBS
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Williams and Zuzel (1985) cloned acylase genes from *E. coli* ATCC 9637 and a mutant (*broA*) differing from the wild-type enzyme in its preference for the substrate N-(3-carboxy-4-nitrophenyl)-6-bromoadipamide over NIPAB. Replacement subcloning localized the mutation to within a 480 base-pair fragment which had lost a unique restriction site (*Nco*I) in an A to T mutation converting Met to Leu. Subsequent sequencing (Schumacher *et al.*, 1986) identified such a site in the gene from strain ATCC 11105 (PACEC) as Met168 (position 182, *Table 3*). Activity towards N-(3-carboxy-4-nitrophenyl)-6-bromoadipamide was also found in mutants containing Val, Ala, Thr, Gly or His at this position; all of these mutants were also active towards NIPAB. Martín *et al.* (1990a) have found differences in the thermal stability and in Arrhenius plots for k_{cat} and k_{cat}/K_m in the Met168Ala mutant obtained from *K. citrophila*.

Forney, Wong and Ferber (1989), using random mutagenesis of the cloned gene of *E. coli* ATCC 11105, selected mutant enzymes with the ability to hydrolyse glutaryl-L-leucine more efficiently than the wild-type enzyme. In a similar study, Forney and Wong (1989) obtained a tenfold increase in the catalytic efficiency (k_{cat}/K_m for the purified enzymes) for the hydrolysis of D-(−)-α-aminophenylacetyl-L-leucine, which was chosen as an analogue of ampicillin and cephalexin.

FLANKING REGIONS

Some sequence patterns have been recognized which appear to correspond with sites of importance in transcription and translation.

Transcription

Valle *et al.* (1986), using extension of a DNA primer which hybridized within the protein-coding region and RNA isolated from cells expressing the *E. coli* gene, determined the transcriptional start point as 31 ± 1 nucleotides from the translational start point. Putative −35 (TAGATA) and −10 (TAGTAT) sequences were identified as similar to the consensus sequences and these sequences were separated by 17 bp, a distance optimal for promoter activity. A putative terminator sequence with two inverted repeats has been found in the flanking sequence beyond the C-terminal stop codon, TAA (Guo *et al.*, 1989).

Translation

The purine-rich sequence motif GAGGA beginning 8 bp upstream of the initiator codon in *E. coli* (Schumacher *et al*, 1986; Guo *et al.*, 1989) was presumed to contain a Shine–Dalgarno sequence recognized in the binding of mRNA to the ribosome. A similar putative binding sequence, AAGAGG, was found 11 bp upstream of the initiation codon in *K. citrophila* (Barbero *et al.*, 1986). In three genes from pseudomonal strains, the comparable sequence GAGG has been noted (Matsuda and Komatsu, 1985; Matsuda, Toma and Komatsu, 1987).

In *E. coli*, the biosynthesis of penicillin acylase is subject to glucose catabolite repression mediated through cyclic AMP (Gang and Shaikh, 1976). Valle *et al.* (1986) have identified two potential cyclic AMP receptor protein-binding sites, both homologous with the consensus sequence.

MODIFICATION OF REGULATION OF EXPRESSION

Daumy, McColl and Apostolakos (1982) found that the biosynthesis of the enzyme from *P. rettgeri* was subject to a different mode of regulation from that in *E. coli*; the enzyme was not induced by phenylacetic acid and not subject to catabolite repression by glucose. Instead, the apparently constitutive expression was subject to repression by succinate, fumarate and malate. The *E. coli* pattern of catabolite repression regulation was found when the *P. rettgeri* penicillin acylase gene was expressed in *E. coli* (Daumy *et al.*, 1986). When the growth of the organism depended on different amide substrates as sole nitrogen source, a variety of mutants of *E. coli* and *P. rettgeri* were generated in which the penicillin acylase gene was either deregulated or modified to give new substrate specificities (Daumy *et al.*, 1985). Enhanced levels of enzyme production in *E. coli*, presumably through deregulation of control of the gene, have also been obtained by conventional mutagenesis (Kochetkova *et al.*, 1986). Acylase biosynthesis in *A. viscosus*, like that in *E. coli*, is induced by phenylacetic acid but this regulation was lost when the cloned gene was expressed in either *E. coli* or *Bacillus subtilis* and expression became constitutive (Ohashi *et al.*, 1989). When penicillin acylase from overproducing strains of *E. coli* and *B. megaterium* were cloned and expressed in *E. coli*, the production of the recombinant protein was constitutive and higher than in the original strains (Meevootisom and Saunders, 1987) but the recombinant plasmids were unstable, recalling an earlier report on plasmid instability in *E. coli* (Deretic, Francetic and Glisin, 1984). Expression of the *B. sphaericus* gene in *B. subtilis* was enhanced twofold, but expression in whole cells of *E. coli* was low (Olsson *et al.*, 1985). However, expression was enhanced 200-fold when the gene was inserted so as to be induced under the control of the *lac* promoter (Olsson and Uhlén, 1986).

Maturation

Present evidence for the *E. coli* ATCC 11105 enzyme supports the view that removal of the signal peptide is the first step in the processing pathway and that the specific cleavage at the N-terminus of the β-subunit occurs after the precursor has traversed the cytoplasmic membrane. Other cleavage events discussed below are apparently less specific and may occur either in the cytoplasmic or in the periplasmic spaces.

Böck *et al.* (1983a) used immunoblotting to analyse total cell lysates of *E. coli* strains harbouring the penicillin acylase gene from strain ATCC 11105 on plasmid pHM12. In addition to α- and β-subunits, a large polypeptide was detected with a molecular weight approximately equal to the sum of the molecular weights of the α- and β-subunits. A similar polypeptide was found

as a product of *in vitro* translation. Böck *et al.* (1983b) showed that deletion of the DNA segment coding for the penicillin acylase resulted in lack of synthesis of the large polypeptide, as well as of α- and β-chains. This evidence, suggesting the large protein to be a precursor, was supported by the results of limited digestion of the putative precursor; the same pattern of molecular weights was found in the resulting peptides as in the peptides obtained by digestion of the α- and β-subunits. Additionally, there was a decrease in recovery of mature subunits after inhibition of protein synthesis by chloramphenicol. Immunoblotting of cellular fractions of cells harbouring plasmid pHM12 showed the precursor to be associated almost exclusively with cell membranes. These results suggested that the processing of the precursor is coupled with release of the mature subunits into the periplasmic compartment. A pointer to one aspect of the complexity of processing was the observation that the molecular weight of the α-subunit isolated from cell lysates was slightly greater than the molecular weight of the α-subunit in the mature enzyme. This became explicable when sequencing demonstrated the existence of an endopeptide (Bruns *et al.*, 1985; Schumacher *et al.*, 1986).

ROLE OF PRECURSOR STRUCTURE

Sizmann, Keilmann and Böck (1990) have investigated the effects of mutation in different parts of the penicillin acylase gene on the ability of the cell to synthesize the precursor and to process it to produce active enzyme in the periplasm. For these experiments, polyclonal antisera were raised against the penicillin acylase holoenzyme, the α-subunit and the β-subunit, and Western blotting was used to identify the protein products in SDS-lysates separated by SDS-polyacrylamide gel electrophoresis.

Endopeptide

Insertion of the tetrapeptide Ala–Asp–Pro–Arg within the endopeptide allowed specific cleavage during processing, although at reduced efficiency: a preponderance of precursor was found in cell lysates, together with some α- and β-subunits; and cell-free extracts contained about 40% of the catalytic activity of controls. This shows that some disturbance to the structure of the endopeptide region can be tolerated. However, extension of the endopeptide by 24 amino acids blocked processing and a stable precursor accumulated in the cell. Deletion of the N-terminal 43 residues of the endopeptide (leaving only 11 endopeptide amino-acid residues) resulted in a precursor susceptible to unspecific proteolytic cleavage, so that several smaller protein fragments were detected by antibody binding (Sizmann, Keilmann and Böck, 1990).

C-terminal region

The insertion of the tetrapeptide Ala–Asp–Pro–Arg near the C-terminus of the precursor completely blocked the formation of active enzyme and no

α- and β-subunits were detected. The precursor was found exclusively associated with the membrane fraction, suggesting a defect in translocation. This interpretation was supported by the effects of deletion of C-terminal residues. A precursor lacking three residues was processed, although there was a decrease in the amount of precursor synthesized. Deletion of 6, 9, 11 or 19 C-terminal residues completely blocked specific post-translational proteolytic cleavage of the endopeptide. Western blotting analysis of the mutant lacking 19 residues showed that the signal peptide was cleaved off, implying at least partial translocation of the protein. In all of these mutants there was evidence of non-specific proteolytic cleavage of the precursor, suggesting that an abnormal protein-folding pathway may lead to exposure of normally inaccessible peptide bonds (Sizmann, Keilmann and Böck, 1990).

Internal positions in α- and β-chains

When 19 amino-acid residues were deleted from within the α-chain (positions 95–113) the protein was detected only in trace amounts, perhaps owing to its conformational instability and rapid proteolytic degradation. The effect of deletion of even large numbers of residues (either 64 residues from positions 477–540, or 334 residues from positions 477–810) from within the β-subunit was different in that a stable protein product was formed by cleavage of the signal peptide from the precursor (Sizmann, Keilmann and Böck, 1990).

Absence of a specific protease

Transformation of several enterobacteria and *Pseudomonas putida* with plasmids bearing the *pac* gene demonstrated that post-translational modification occurred in all of the tested organisms (Sizmann, Keilmann and Böck, 1990). This heterologous expression argues against the existence of a specific protease concerned with the processing of penicillin acylase. One possibility is that conformational features of the folded precursor, after cleavage of the signal peptide, can be recognized by a variety of proteases. Specific autocatalysis after translocation also remains a possibility.

Fewer results have been reported for other enzymes, but the presence of a variety of N-terminal residues arising from partial cleavage at several neighbouring positions in the α-subunit of the *A. viscosus* enzyme (Ohashi *et al.*, 1988) is suggestive of the potential involvement of more than one protease.

Order of processing steps

Schumacher *et al.* (1986) found that a precursor lacking the signal peptide remained in the cytoplasm but was cleaved to yield free subunits when the cells were disrupted. There was an initial cleavage at the N-terminus of the β-subunit, a second cleavage within the endopeptide and a final cleavage to free the C-terminus of the α-subunit. Sizmann, Keilmann and Böck (1990) used monospecific antisera directed against the purified subunits to detect the

formation *in vivo* of a species recognized by anti-α-antibodies and possessing a molecular weight corresponding to that of the α-subunit plus the endopeptide. A pulse-chase experiment showed a precursor–product relationship between the putative intermediate and the mature α-subunit.

Further evidence for the composition of the intermediate was obtained by immunoprecipitation of ^{35}S-labelled protein after expression of a precursor gene lacking the region coding for the signal peptide. The precipitated protein co-migrated in SDS-polyacrylamide gel electrophoresis with the intermediate (α + endopeptide) obtained from the normal precursor. This experiment showed that the precursor can be partly processed in the cytoplasm because it had previously been shown (Schumacher *et al.*, 1986) that the mutant precursor lacking the signal peptide could not be exported.

Comparison with other proteins

Sizmann, Keilmann and Böck (1990) have pointed out that, although proteolytic post-translational processing has been found in several bacterial proteins, there are no well-characterized cases which closely resemble the processing of bacterial penicillin acylase. For example, maturation of the cytochrome bc_1 complex from *Bradyrhizobium japonicum* into its individual subunits involves processing which is not a prerequisite for function (Thöny-Meyer, Stax and Hennecke, 1989). In *B. polymyxa*, a precursor protein is cleaved to give two separate enzymes, α- (EC 3.2.1.1) and β-amylase (EC 3.2.1.2) (Uozumi *et al.*, 1989). Cleavage of the pro-protein of *B. subtilis* subtilisin (EC 3.4.21.14) is necessary to yield the active enzyme (Ikemura, Takagi and Inouye, 1987) but the pro-sequence product does not form a part of the active enzyme, although it acts as a chaperone for correct folding (Power, Adams and Wells, 1986). The intermolecular autocatalytic mechanism reported for the subtilisin pro-protein (Zhu *et al.*, 1989) has not been demonstrated in penicillin acylase, although, as noted above, autocatalysis has not been ruled out.

Solution properties

CONFORMATION

Similar circular dichroism spectra for the enzyme from *K. citrophila* (Márquez *et al.*, 1988) have been obtained over a range of temperature, pH and salt concentration. The far-UV spectra were interpreted in terms of α-helix (11%), β-sheet (44%) and β-turn (11%) but it should be noted that estimates of the β-turn content of proteins pose considerable difficulties.

The *E. coli* ATCC 11105 enzyme is globular and close to spherical in shape in aqueous solution (Lindsay and Pain, 1990a): the sedimentation constant $s_{20,w} = 5·95$S and the partial specific volume $\bar{v}_2 = 0·739$ calculated from the amino-acid composition led to a frictional ratio $f/f_0 = 1·13$, among the lowest values measured for a range of globular proteins. This is more symmetrical

than implied by values reported for the *B. megaterium* enzyme (Chiang and Bennett, 1967): the sedimentation coefficient was 5·5S and the apparent molecular mass estimated by rapid equilibrium sedimentation was approximately 120 000 Da. The α-helix and β-sheet contents of the native *E. coli* enzyme were estimated from analysis of far-UV circular dichroism to be 24% and 57%, respectively. The near-UV circular dichroism spectrum showed marked positive ellipticity close to 100 deg cm^2 dmol^{-1} between 260 and 270 nm, with substantial contributions from tryptophan, tyrosine and phenylalanine residues, characteristic of a well-defined tertiary structure (Lindsay and Pain, 1990a).

STABILITY AND DENATURATION

Until recently, the stability of penicillin acylase has been discussed mainly in relation to effects on the rate of loss of activity. For example, Berezin *et al.* (1975) used the inactivation of enzyme activity by ultrasound to probe enzyme stability as a function of pH and temperature. More recently, Andersson and Hahn-Häagerdal (1987a) showed that the half-life of enzyme activity correlated with the transition temperature determined by differential scanning calorimetry, and these parameters were used to determine the stabilizing effects of poly(ethyleneglycol) and potassium phosphate. The stability did not correlate with water activity but was related more to solute concentration. These solutes also decreased the enzyme activity and, in the case of poly(ethyleneglycol), the decrease correlated with water activity (Andersson and Hahn-Häagerdal, 1987b).

The reversibility of urea-denaturation of the *E. coli* ATCC 11105 enzyme has been studied by a number of physical techniques (Lindsay and Pain, 1990a). The enzyme unfolded co-operatively over a narrow range of urea concentration, with a mid-point at 4·5 M urea, the normalized unfolding curves being superimposable for fluorescence and enzyme activity (monitoring tertiary structure), and far-UV circular dichroism (monitoring secondary structure). The unfolding kinetics were strongly dependent on urea concentration, which is typical of co-operatively structured proteins. Urea-gradient gel electrophoresis demonstrated that the separated β-peptide readily aggregates whereas the α-peptide refolds reversibly to a compact state.

The physical properties (Stokes radius, sedimentation coefficient and frictional ratio) of the refolded α-peptide were indicative of a compact, asymmetric structure. Compared with the native enzyme there was greater sensitivity to urea-denaturation with a mid-point at 3·8 M urea and, assuming a two-state transition, a free energy of stabilization of the folded form in the absence of denaturant, $\triangle G_0$, of 11·8 kJ mol^{-1}. This relatively low value implies that one molecule in a hundred is in the unfolded state. The far-UV circular dichroism of the refolded α-peptide indicated substantially more secondary structure (32% α-helix and 48% β-sheet) than in the native enzyme, and the near-UV circular dichroism spectrum was characteristic of a well-defined tertiary structure, but the main features of the spectrum differed markedly from those of the native enzyme, with only a small contribution

from tryptophan residues. Quenching of fluorescence by both iodide and caesium ions was less with the α-peptide than with the native enzyme, indicating that a smaller proportion of tryptophan residues are accessible to quenching in the α-peptide. Evidence for the existence of a hydrophobic patch on the surface of the folded α-peptide was obtained using the fluorescent probe, 8-anilino-1-naphthalene sulphonic acid; the fluorescence was strongly enhanced in the presence of the α-chain, while the native enzyme, like many globular proteins, bound little or none. Furthermore, measurement of static anisotropy of the bound dye showed that the binding was to a limited site in the folded α-peptide.

On the basis of these results, it was suggested (Lindsay and Pain, 1990a) that the α-peptide constitutes a folding domain which, *in vivo*, provides a folded structure onto which the β-peptide can subsequently fold. This mechanism would avoid the aggregation of the β-peptide which readily occurs *in vitro*.

REFOLDING AND ASSEMBLY

Lindsay and Pain (1990b) have investigated the effects of pH, ionic strength and temperature on the assembly of active penicillin acylase from the unfolded α- and β-peptides. Under the optimal conditions found, yields of more than 50% of active enzyme were obtained from separated and unfolded α- and β-peptides. The presence of a molar excess of folded α-peptide enhanced assembly, confirming its effectiveness in the competition between aggregation and assembly of the β-peptide. These findings provide a basis for the assembly of active enzyme by expression of fragments of the gene encoding only the sequences of the mature subunits (Boehringer Mannheim GmbH, 1988).

Catalytic properties

The results of steady-state kinetic and chemical modification studies on penicillin G acylases are consistent with an acyl enzyme mechanism, involving an active site serine in the β-subunit. Recent results identify the N-terminal serine residue as a candidate for the active site nucleophile (Martín *et al.*, 1990b; Slade *et al.*, 1990).

STEADY-STATE KINETICS

Determination of the kinetic parameters has been complicated by the effects of product inhibition. If products accumulate, especially phenylacetic acid in the case of penicillin G acylases (where $K_i > K_m$), initial velocity measurements will be affected such that K_m and V_{max} will be overestimated. For an explanation of the theory see Koerber and Fink (1987). These parameters and K_i for phenylacetic acid have probably been considerably overestimated in some studies (Chiang and Bennett, 1967; Balasingham *et al.*, 1972; Szewezuk, Siewinski and Slowinska, 1980). Better values of K_m have been obtained for

the hydrolysis of benzylpenicillin by *E. coli* penicillin G acylase in the pH range 7–8·5: 2×10^{-5} M (Kutzbach and Rauenbusch, 1974); $4·6 \times 10^{-6}$ M (Berezin *et al.*, 1974); and $8·5 \times 10^{-6}$ M (Veronese *et al.*, 1981). In these studies, values of K_i for phenylacetic acid were in the range $2·8 \times 10^{-5} - 2 \times 10^{-4}$ M. Inhibition by 6-APA is much weaker ($K_i \approx 10^{-2}$ M) and approximately non-competitive.

It was noted by Konecny (1981) that the ability of penicillin acylase to catalyse acyl group transfers which include *trans*-acylation could be explained readily by the formation of an acyl enzyme intermediate. Phenylacetic acid would be predicted to be a competitive inhibitor and, assuming direct attack of the nucleophile on the acyl enzyme, 6-APA would be a non-competitive inhibitor. A similar mechanism was proposed for α-amino acid ester hydrolase (Kato, 1980).

Margolin, Svedas and Berezin (1980) combined active site titration (see the following section) and steady-state kinetics of hydrolysis of 12 different substrates of *E. coli* penicillin acylase. Benzylpenicillin gave the largest value of k_{cat}/K_m (10^7 M^{-1} s^{-1}). Most of the substrates with a phenylacetyl group in the acyl moiety showed similar values of k_{cat} (close to 50 s^{-1}) but the k_{cat} for esters of phenylacetic acid was about threefold greater. This could imply a difference in the rate-limiting step for catalysis, with acylation limiting the turnover of amides and anilides and deacylation limiting the turnover of esters. The pH-dependence of k_{cat}/K_m for phenylacetic and D-(–)-α-aminophenylacetic acid *p*-nitroanilides was consistent with binding of the deprotonated form of the substrate.

CHEMICAL MODIFICATION

The enzyme is inhibited by a number of group-directed reagents, but results with these reagents are more difficult to interpret than those with site-directed reagents, owing to the greater risk of indirect modes of inhibition. Among studies summarized by Mahajan (1984), tryptophan has been suggested to be an essential residue (Mahajan and Borkar, 1983).

Irreversible inactivation of penicillin acylase by an equimolar concentration of phenylmethanesulphonyl fluoride (PMSF) was observed by Kutzbach and Rauenbusch (1974). The kinetics of inactivation (Shvyadas *et al.*, 1977) indicated that binding of the inhibitor occurred prior to the inactivation step and that there was competition between inhibitor and substrate binding. The inactivation was reversible in the presence of a low concentration of the reaction product, 6-APA, which was presumed to act as a nucleophile in the displacement of the sulphonyl derivative. Measurement of residual enzyme activity after reaction with the inhibitor was therefore proposed as the basis for active site titration of the enzyme, in either soluble or immobilized preparations.

As well as PMSF, other phenylmethanesulphonyl compounds, such as the chloride, the azide and the *N*-hydroxysuccinimide ester, have been shown to be inhibitors (Siewinski, Kuropatwa and Szewczuk, 1984).

Evidence that the PMSF-reactive residue is in the β-chain of the enzyme

from *P. rettgeri* was obtained by Daumy, Danley and McColl (1985). After treatment with PMSF, the completely inactive and urea-denatured protein was mixed with denatured normal α- or β-subunits (neither of which possessed any catalytic activity) and then dialysed against an aqueous buffer. Activity was recovered only when untreated β-subunits were present. It is plausible to conclude (Kutzbach and Rauenbusch, 1974) that the effectiveness of PMSF as an inhibitor is associated with its chemical similarity to the phenylacetyl moiety of a substrate, leading to site-directed modification of an active site residue by placing the reactive sulphonyl group in a position close to that occupied by the carbonyl group of an amide or ester substrate. A serine residue may be suggested as an active site nucleophile by analogy with serine proteases and in view of kinetic evidence favouring the existence of an acyl enzyme intermediate. Cysteine would be an alternative target for PMSF but this can be ruled out in the case of the *E. coli* enzyme, cysteine being absent from the mature enzyme (Schumacher *et al.*, 1986). Kutzbach and Rauenbusch (1974) found little inhibition with di-isopropylphosphofluoridate or with the chymotrypsin inhibitor, L-1-chloro-4-phenyl-3-*p*-tosylamido-2-butanone, but steric factors may explain these results.

Recently, the 'chemical mutagenesis' reaction used to convert the subtilisin active site serine to cysteine (Neet and Koshland, 1966) has been applied to the enzymes from *E. coli* ATCC 11105 (Slade *et al.*, 1990) and *K. citrophila* (Martín *et al.*, 1990b). In each case, the β-subunit N-terminal serine (*Table 3*, position 305) was converted to cysteine. The resulting thiol-acylase possessed little or no catalytic activity, although near-UV circular dichroism provided no evidence of change in tertiary structure. The thiol-group reactivity in the modified *E. coli* enzyme was consistent with an environment with hindered accessibility to solvent. On the simplest interpretation, these results make the β-subunit N-terminal serine residue a candidate for an active site nucleophile. This would imply that penicillin acylase belongs to a new class of serine enzymes; such a position for an active site serine appears to be unprecedented.

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