Purification and preliminary crystallographic studies of penicillin G acylase from *Providencia rettgeri*

HERBERT E. KLEI,¹ GASTON O. DAUMY,² AND JUDITH A. KELLY¹

¹ Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269-3125

² Pfizer Central Research, Groton, Connecticut 06340

(RECEIVED November 2, 1994; ACCEPTED January 6, 1995)

Abstract

Two isoforms of the heterodimeric enzyme penicillin G acylase (EC 3.5.1.11) from *Providencia rettgeri* ATCC 31052 (strain Bro1) were purified to near homogeneity. The isoforms exhibited comparable enzymatic activities but differed slightly in the molecular weight and pI of their respective α -subunit. The origin of this difference was traced to the partial conversion of the N-terminal Gln of the α -subunit to pyrrolidonecarboxylic acid (pyro-Glu). The boundaries of the mature enzyme within the translated DNA sequence of the wild-type propeptide (GenBank M86533) were determined. The results conclusively identified the length of the signal peptide and the position of the spacer cleaved from the propeptide to form the active heterodimer. The molecular weights of the α - and β -subunits, based on these termini, were 23.7 and 62.2 kDa, respectively. Both isoforms were crystallized independently as hexagonal bipyramids up to 0.60 mm in diameter in either space group P6₁22 or P6₅22 ($a = b = 140.5$ Å and $c = 209.5$ Å) from ammonium sulfate solutions buffered by 50 mM potassium phosphate at pH 7.5. The presence of glycerol, although not required, facilitated crystal growth. Native and heavy atom derivative data were collected to 3.0 Å resolution, and the calculation of isomorphous replacement phases is under way.

Keywords: enzyme purification; penicillin G acylase; protein crystallization; *Providencia rettgeri*; pyrrolidonecarboxylic acid (pyro-Glu)

β -Lactam antibiotics, which include penicillins and cephalosporins, have been widely used in the treatment of infections for more than 50 years. These drugs are well suited for this purpose because there is no mammalian counterpart to the target D-alanyl-D-alanine carboxypeptidase/transpeptidase enzymes essential to bacterial cell wall biosynthesis (Frère et al., 1992). The long-term effectiveness of β -lactams as antibacterial drugs is threatened by the proliferation of bacterial resistance to these drugs. The most common mechanism by which bacterial strains acquire resistance is through the transfer of plasmids carrying the genes for resistance enzymes. The resistance enzymes, known as β -lactamases, rapidly hydrolyze the β -lactam bond, rendering the drugs useless as antibacterial agents (Waley, 1992). Resistance to specific and historically effective β -lactam compounds can also be gained through mutation of the DD-peptidase genes (Schultz et al., 1991). Pharmaceutical companies must continually develop and synthesize new and more effective antibiotics to counter the spread of bacterial resistance. De novo synthesis of β -lactams is difficult and costly; therefore, the enzyme penicillin G acylase is valuable in the commercial manufacture of

penicillins. This enzyme catalyzes the hydrolysis of the common and inexpensive antibiotic penicillin G into phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA). The 6-APA is subsequently used in the synthesis of new penicillins with broader inhibitory profiles and greater effectiveness. Unfortunately, penicillin G acylases are not able to readily produce 7-aminocephalosporanic acid (7-ACA) from cephalosporin C by the analogous enzymatic reaction.

The penicillin G acylase propeptide from *Providencia rettgeri* (formerly *Proteus rettgeri*) is proteolytically processed much like the penicillin G acylases found in *Escherichia coli* and *Kluyvera citrophila* (Daumy et al., 1985b; Barbero et al., 1986; Schumacher et al., 1986). Posttranslational maturation begins with the cleavage of a signal peptide after membrane translocation. This cleavage is followed by the removal of a spacer from between the α - and β -subunits, which is unusual for proteins of prokaryotic origin. Successful processing and the formation of the $\alpha\beta$ heterodimer are required to produce active periplasmic enzyme (Piotraschke et al., 1994). Substrate specificity generally resides in the α -subunit (Daumy et al., 1985a); however, recent work (Roa et al., 1994) also attributes some specificity to the β -subunit. The N-terminal phenylmethylsulfonyl fluoride-sensitive serine residue required for catalytic activity is contained in the β -subunit (Slade et al., 1991; Choi et al., 1992).

Reprint requests to: Judith A. Kelly, Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269-3125; e-mail: kelly@uconnvm.uconn.edu.

Substrate specificity is primarily attributed to the lactam side chain and not to differences between the 6-APA 5-membered and 7-ACA 6-membered ring moieties (Huang et al., 1963; G.O. Daumy & A.S. McColl, unpubl. results). Wild-type *P. rettgeri* was subjected to sequential steps of selective pressure based on the ability to use hexanamide, followed by 6-bromohexanamide (BrHEX; Fig. 1), as the only nitrogen source (Daumy et al., 1985b). The penicillin G acylase activity of the mutant strain, referred to as Bro1, exhibited markedly different enzyme kinetics from the parental strain. The change in specificity achieved through spontaneous mutation suggested that further selection with amides that more closely resembled the lactam side chain of cephalosporin C (labeled R_2 in Fig. 1) might eventually yield mutant strains able to deacylate cephalosporin C. Cephalosporin C acylase activity, once gained, could then be employed to produce 7-ACA, which in turn could be used to manufacture semi-synthetic cephalosporins. Unfortunately, mutants harboring this activity were not isolated. The difference between R_2 and BrHEX is the amino and carboxyl groups on the zwitterionic carbon of R_2 , indicating that further steric and electrostatic modification of the penicillin G acylase active site was likely required. Several attempts to introduce charge compatibility into the active site by positive selection with 6-carboxylhexanamide (CbHEX; Fig. 1) as the nitrogen source were unsuccessful (G.O. Daumy & A.S. McColl, unpubl. results). In view of the inability to acquire cephalosporin C acylase activity through mutation, the emphasis of the work shifted toward the determination of the three-dimensional structure of the enzyme. The long-range goals are to elucidate the hydrolysis mechanism used to cleave hydrophobic moieties (such as R_1 in Fig. 1) and to use this information to guide the modification of the penicillin acylase active site to accept more hydrophilic charged moieties (such as R_2 in Fig. 1). Reports on the purification and crystallization of penicillin G acylase from *E. coli* are available (Kutz-

bach & Rauenbusch, 1974; Hunt et al., 1990), but there is no published crystal structure.

Two active isoforms of penicillin G acylase from *P. rettgeri* (strain Bro1) were observed with the established purification protocol (Daumy et al., 1985b); however, the doublet was never sufficiently resolved to permit the separation of the two peaks (Bourque, 1988). Rather, fractions were assigned ratios (40:60, for example) based on the percentage area of the first peak relative to the second peak. Numerous attempts to grow crystals from these fractions, whose purities ranged from 75% to 92% acylase, were unsuccessful. Excessive impurities, and possibly the mixture of the two isoforms, were thought to be responsible for this difficulty. In this communication, we describe an improved purification protocol, the successful crystallization of the enzyme, the determination of the boundaries of the α - and β -subunits within the translated DNA sequence of the wild-type propeptide (Ljubijankic et al., 1992), and preliminary X-ray crystallographic results.

Results

Use of an additional anion exchange step by fast protein liquid chromatography (FPLC/MonoQ 10/10) to augment the established isolation protocol (Daumy et al., 1985b) resolved the preparation into two protein peaks. Although baseline separation was not achieved, it was possible to isolate samples of each peak by the judicious collection and combination of fractions. The salt gradient used to elute the protein was very gradual (0.1% of 1 M NaCl/min). The first peak consistently eluted at 1% lower salt concentration than the second peak (an average of 7.9% versus 8.9% salt). Three protein preparations were maintained: P1, which predominantly contained protein from the first peak; P1P2, which contained protein from the overlap of the two peaks; and P2, which predominantly contained protein

Fig. 1. Structures of penicillin and cephalosporin with R_1 , R_2 , and L used as abbreviations for the variable groups. Specific examples of the variable groups for penicillin G (R_1) and cephalosporin C (R_2 and L = $-\text{OCOCH}_3$) are included. Structures of BrHEX and CbHEX are also shown for comparison with R_1 and R_2 . Solid and dashed wedges represent chiral centers in the R and S configurations, respectively.

from the second peak. The P1 and P2 preparations appeared nearly homogeneous, with minimal cross-contamination when aliquots were rechromatographed on an analytical FPLC/MonoQ 5/5 column. The P1P2 aliquot, as expected, indicated the presence of protein from both the P1 and P2 preparations. All three FPLC/MonoQ 5/5 elution profiles are shown in Figure 2. Final purification yielded 16.4 mg of protein (15.0 mg of P1 and 1.4 mg of P2) from 250 mL of frozen cell paste.

The P1 and P2 preparations were shown to clearly represent different isoforms of penicillin G acylase rather than contamination by unrelated protein. The specific activities of the P1, P1P2, and P2 preparations were the same within experimental variation and ranged between 8 and 12 $\mu\text{mol}/\text{min}$ per mg of protein. All three preparations appeared homogeneous for acylase by SDS-PAGE and yielded bands at the usual apparent molecular weights (M_r) for the α - and β -subunits ($\alpha \approx 24.5$ kDa and $\beta \approx 66.0$ kDa). The P1, P1P2, and P2 preparations were also analyzed by two-dimensional (2D) gel electrophoresis (isoelectric focusing [IEF]/SDS-PAGE). The entire P1 2D gel, along with inserts of key regions from the P1P2 and P2 2D gels, is shown in Figure 3. All preparations exhibited identical M_r values for their respective α - and β -subunits ($\alpha \approx 21.0$ kDa and $\beta \approx 69.4$ kDa), but differed in the pI distribution of their subunits. P1 was enriched in the high pI form of the α -subunit (pH 4.95), whereas P2 was enriched in the low pI form (pH 4.75). This pI difference was consistent with the order in which the two

Fig. 2. Elution profiles of samples from the final FPLC/MonoQ 10/10 purification step rechromatographed through an analytical FPLC/MonoQ 5/5 column. Minimal cross-contamination between P1 and P2 preparations was seen. The P1P2 preparation, taken from fractions where P1 and P2 were not resolved, showed both isoforms, as expected. The P1P2 curve was translated to an earlier time by 1 min to align the peaks from the three runs.

Fig. 3. IEF/SDS-PAGE (2D) gel of the P1 preparation with insets from the 2D gels of the P1P2 and P2 preparations. Insets are placed at the correct pH (horizontally) but offset vertically to allow easier comparison of the α - and β -subunit regions from the three 2D gels. M_r values of the α - and β -subunits were determined to be 21.0 and 69.4 kDa, respectively. No difference in M_r between the different forms of either subunit could be detected from the 2D gels. Differences of 0.20 and 0.07 pH units were measured between the pIs of the two forms of the α - and β -subunits, respectively. The positions of these molecular weights and pIs are labeled along the appropriate axes by inner tick marks.

isoforms of the intact heterodimer eluted from the anion exchange columns. The β -subunit from the P1 preparation appeared homogeneous with respect to pI, but another low pI form was detected in the P2 preparation. The pIs of the two forms of the β -subunit were measured to be 6.98 and 6.91. Electrospray mass spectrometry (ESMS) of the P1 and P2 samples measured the molecular weights of the high and low pI forms of the α -subunit to be $23,715.4 \pm 1.4$ Da and $23,700.0 \pm 1.2$ Da, respectively. Unfortunately, the β -subunit was not seen by ESMS because its mass bordered on the upper limit of the instrumental setup.

The purity of the P1 preparation was measured to be greater than 98% based on peak areas observed by FPLC/MonoQ and capillary zone electrophoresis (CZE). The P2 preparation was measured to be slightly less pure, 96% based on FPLC/MonoQ peak areas. The higher purity of the P1 preparation relative to the P2 preparation was further substantiated by the presence of increased impurities in the P2 2D gel compared to the P1 2D gel. From these protein characterization studies (SDS-PAGE, FPLC/MonoQ, IEF/SDS-PAGE, and ESMS), we concluded that the P1 and P2 penicillin acylase preparations were greater than 96% pure, the differences between the α -subunit of the two active isoforms were small (0.2 pH units and 15.4 Da), and the differences between the isoforms resided primarily in the α -subunit (Table 1).

Because one of the objectives of this work was to determine the atomic-level structure of penicillin G acylase from *P. rettgeri* (strain Bro1), the primary sequence of the mature enzyme was needed to help with the interpretation of the electron density. The P1P2 preparation was used to determine partial N-terminal sequences in order to conserve the P1 and P2 preparations for crystallization trials. The α - and β -subunits from

Table 1. Summary of subunit differences between the two isoforms of penicillin G acylase (strain Bro1)

		pI		M_r (Da)	
		2D gel	Calc. ^a	ESMS	Calc. ^a
α Subunit	Form 1	4.95	4.51	23,715.4 $\pm$ 1.4	23,732.3
	Form 2	4.75		23,700.0 $\pm$ 1.2	
β Subunit	Form 1	6.98	6.13	Not seen	62,156.0
	Form 2	6.91		Not seen	
				Total	85,888.3

^a Calculated values were computed by the PEPTIDESORT routine (Genetics Computer Group, Wisconsin Package, version 8.0 OpenVMS) and are based solely on the wild-type primary sequence (GenBank M86533).

the PIP2 preparation were separated by SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membrane, and the N-terminal residues of each subunit were sequenced. No sequence was obtained from the blot of the α -subunit, whereas 38 of the first 42 N-terminal residues of the β -subunit were clearly identified. In order to obtain larger quantities of the α -subunit, PIP2 acylase was first dissociated in guanidine hydrochloride (GnHCl). The α -subunit was then isolated by size

exclusion chromatography, refolded by dialysis against water, lyophilized, and sequenced. The signal from the first Edman degradation cycle was too low to provide conclusive identification of the first amino acid; however, the next 46 residues of the α -subunit were unambiguously identified. The homology between the N-terminal sequences of *P. rettgeri* (strain Bro1) and the equivalent *E. coli* and *K. citrophila* sequences was 75%. Fortunately, the sequence of the entire penicillin G acylase gene of wild-type *P. rettgeri* then became available as GenBank entry M86533 (Ljubijankic et al., 1992). The N-terminal sequences identified by this work are positioned within the translated DNA sequence in Figure 4.

Alignment of our N-terminal sequences with the translated DNA sequence suggested that the N-terminal residue of the α -subunit was Gln. N-terminal Gln and Glu are known to be susceptible to the formation of pyrrolidonecarboxylic acid (pyro-Glu) (Orlowski & Meister, 1971). The difficulty experienced with determination of the α -subunit sequence was consistent with partial blockage of the α -subunit in the PIP2 preparation. The lower pI (0.20 pH units) and lower molecular weight (15.4 $\pm$ 2.6 Da) observed for the P2 α -subunit were also consistent with the loss of an amino group as the result of cyclization. Several experiments were conducted to confirm that pyro-Glu was responsible for the different forms of the α -subunit. The P2 acylase was digested with pyroglutamate aminopeptidase (EC 3.4.19.3), which removes only N-terminal pyro-Glu residues. Subunits were then separated by SDS-PAGE, blotted, and individually

		F signal			
1		MKKH LISIAIVLSL			
		↓			
15	SSLSLSSFSQ	STQIKIERDN	YGVPHIYAND	TYSLFYGYGY	AVAQDRLFQM
		X	STQIKIERDN	YGVPHIYAND	TYSLFYGYGY
					AVAQDRLFQM
		↓			
65	EMAKRSTQGT	VSEVFGKDYI	SFDKEIRNNY	WPDSIHKQIN	QLPSQEQDIL
	EMAKRS				
		↓			
115	RGYADGMNAW	IKQINTKPDD	LMPKQFIDYD	FLPSQWTSFD	VAMIMVGTMA
		↓			
165	NRFSDMNSEI	DNLALLTALK	DKYGEQLGVE	FFNQINWLNN	PNAPTTISSE
		↓			
215	EFTYSDSQKT	KNISQLNQIS	DYRLTAPMFE	RTAKDTTGKV	LALSSQENNA
		↓			
265	LIAKQYEQSG	ANGLAGYPTT	SNVWLVGKTK	ASGAKAILLN	GPQFGWFNPA
			SNVWLVGKTK	ASGAKAILLN	GPQFGWFNPA
315	YTYGIGLHGA	GFNIVGNTPF	AYPAILFGHN	GHVSWGSTAG	FGDGVDIFAE
	YTYXIGLXXA	XF			
365	QVSPEDPNSY	LHQGQWKML	SRQETLNVKG	EQPITFEIYR	TVHGNVV...
		↓			
815	PLWLNSDVA	PYIESTETLI	IER		

Fig. 4. Comparison of the translated wild-type *P. rettgeri* penicillin G acylase prepeptide DNA sequence (GenBank M86533) (Ljubijankic et al., 1992) and our partial N-terminal sequences from strain Bro1. Sequences determined by Edman degradation are listed below the DNA translation. Amino acid residues marked as X (one in the α -subunit and four in the β -subunit) were not unambiguously identified. CNBr digest sites are indicated in the α -subunit only by arrows (↓). The symbols □ and □ mark the endpoints of the signal peptide (1–23), α -subunit (24–228), spacer peptide (229–284), and β -subunit (285–837). Residue 1 was assigned to the start of the signal peptide to be consistent with earlier convention (Barbero et al., 1986; Schumacher et al., 1986). Residues 412–814 of the β -subunit are not shown.

sequenced. The sequence of the treated α -subunit began with the second residue (Ser 25), whereas the sequence of the β -subunit showed no change. Filtrate from the centrifugal concentration of the digest mixture was subjected to acid hydrolysis and analyzed by gas chromatography/mass spectrometry (GC/MS). The presence of pyro-Glu at 129.0 ± 0.5 Da was seen as the only peak in both the filtrate and the standard. In the absence of pyroglutamate aminopeptidase treatment, no sequence for the P2 α -subunit was detected. When both P1 and P2 samples were sequenced directly, the P2 sample yielded only the sequence of the β -subunit, whereas the P1 sample yielded simultaneous sequences for the α - and β -subunits, although the signal for the α -subunit was noticeably weaker. When originally purified, the particular P1 sample used for these experiments was shown to contain almost exclusively the unblocked isoform of penicillin acylase; however, when reexamined by analytical anion exchange chromatography (SMART/MonoQ), it was discovered to now contain roughly equal amounts of the blocked and unblocked isoforms. The partial conversion of Gln to pyro-Glu, hence partial blockage of the α -subunit, corroborated the weaker signal for the P1 α -subunit. The α -subunit, when isolated by SDS-PAGE and blotted, was never successfully sequenced from any sample (even ones from the PIP2 and P1 preparations) unless the sample was first treated with pyroglutamate aminopeptidase. Therefore, the conditions under which the SDS-PAGE gel and blot were run were presumed to favor the formation of pyro-Glu; however, attempts to cleanly convert PIP2 to P2 at slightly acidic (Laemmli buffer, pH 6.8) and basic (MilliBlot anode buffer, pH 10.4) conditions at 90 °C failed. In retrospect, these conditions were likely too harsh and brought about the deamidation of susceptible Asn residues (Asn followed by Gly or Ala) and possibly the cleavage of some peptide bonds as well (Doolittle, 1977; Melius & Hubbard, 1986; Daumy et al., 1991). None of the samples from failed conversions were sequenced to test if either the pyro-Glu or protein remained intact.

Sequencing experiments clearly defined the N-termini of the α - and β -subunits of the Bro1 penicillin acylase heterodimer. The C-terminus of the β -subunit was evident from the stop codon; however, the C-terminus of the α -subunit depends on the length of the spacer cleaved from the propeptide. In order to confirm the C-terminus of the α -subunit proposed from computer-assisted homology alignments (Konstantinovic et al., 1994), the α -subunit was purified and subjected to cyanogen bromide (CNBr) digestion, and the fragments were analyzed by ESMS. The potential CNBr cleavage sites in the α -subunit are shown in Figure 4. The predicted and experimental masses of the CNBr fragments are listed in Table 2. Only data for fragments over 1 kDa were collected. All the expected fragments were seen, except for fragment 1. This fragment probably bound irreversibly to the C18 reverse-phase column. The mass of the C-terminal fragment (fragment 9) was consistent with Ser 228 as the C-terminal residue of the mature α -subunit. Masses of the ninth fragment with alternate C-terminal amino acids (Ser 228 $\pm$ 2 residues) are listed in Table 2 for comparison. Based on these results, the signal peptide (Met 1–Ser 23), α -subunit (Gln 24–Ser 228), spacer (Gln 229–Thr 284), and β -subunit (Ser 285–Arg 837) contain 23, 205, 56, and 553 residues, respectively.

A factorial search was used to explore crystallization conditions (Carter & Carter, 1979; Carter, 1990). Both isoforms crystallized as hexagonal bipyramids in space group P6₃22, or its

Table 2. Results of CNBr digestion of the α -subunit to identify the C-terminus

	Fragment	Length (aa)	M_r (Da)		
			Calc. ^a	Exp. ^b	
				Prep. 1	Prep. 2
1	24(Gln)–64(Met)	41	4,790.27	Not seen	Not seen
2	65(Glu)–66(Met)	2	231.23	—	—
3	67(Ala)–121(Met)	55	6,328.95	6,334.1 $\pm$ 2.9	6,326.7 $\pm$ 1.2
4	122(Asn)–136(Met)	15	1,739.97	1,739.1 $\pm$ 0.9	1,739.9 $\pm$ 1.5
5	137(Pro)–157(Met)	21	2,488.76	2,489.9	2,489.0
6	158(Ile)–159(Met)	2	215.27	—	—
7	160(Val)–163(Met)	4	359.40	—	—
8	164(Ala)–170(Met)	7	792.83	—	—
9 ^c	171(Asn)–228(Ser)	56	6,554.19	6,553.5 $\pm$ 0.7	6,554.6 $\pm$ 1.1
9 ⁻²	171(Asn)–226(Asn)	54	6,353.96		
9 ⁻¹	171(Asn)–227(Ile)	55	6,467.11		
9 ⁺¹	171(Asn)–229(Gln)	57	6,682.32		
9 ⁺²	171(Asn)–230(Leu)	58	6,795.48		

^a The final amino acid of fragments 1–8 is actually homoserine lactone instead of Met after reaction with CNBr. Thus, the calculated molecular weights of all but the last fragment are 48.1 Da less than the mass predicted from the amino acid sequence.

^b Experimental values were determined by ESMS. Error bars were calculated from the average of the masses of all detected ions. No error bars could be calculated for fragment 5 because only the +2 ion was observed.

^c Experimental masses of fragment 9 were consistent with Ser 228 as the C-terminal residue. Masses of the 9th fragment with alternate C-terminal amino acids (Ser 228 $\pm$ 2 residues) are listed for comparison.

enantiomorph P6₃22, with $a = b = 140.5$ Å and $c = 209.5$ Å. A photograph of the crystals is shown in Figure 5. The P2 preparation was harder to crystallize, possibly due to its lower purity and/or lower concentration. Crystals from the PIP2 preparation were never grown, although fewer trials were attempted. The first crystals were obtained less than 1 month after the purification and regularly thereafter. Drops yielded crystals as quickly as 2 weeks and as late as 2 years after being set up. The optimal conditions were 50 mM K₂HPO₄, pH 7.5, with drop-to-well concentrations of 20/30, 25/35, and 30/45% saturated ammonium sulfate (SAS) and varying amounts of glycerol (0–20%, v/v). Crystals were grown with greater frequency in drops with glycerol (10–20%, v/v). The crystals can be stored virtually indefinitely in 50 mM K₂HPO₄, pH 7.5, or Tris-HCl, pH 7.5, with 60% SAS, 15–20% (v/v) glycerol, and 0.02% (w/v) sodium azide. All crystals were grown and stored at 18–20 °C. The crystalline enzyme was shown to be active. A crystal was exposed to the substrate 6-nitro-3-phenylacetamidobenzoic acid (NIPAB) dissolved in the K₂HPO₄ storage solution (Kutzbach & Raunbusch, 1974). Within minutes the crystal turned yellow due to the formation of 3-amino-6-nitrobenzoic acid (ANB), whereas the bulk solution remained clear. Gradually, over the course of several hours, the bulk solution also turned light yellow as the product diffused out of and away from the crystal. Four native (two of each isoform) and 15 heavy atom derivative data sets were collected to 3.0 Å at room temperature. These native data sets contained approximately 210,000 observations of 22,000 merged and 40,000 unmerged reflections (95–98%

Fig. 5. Photograph (50 $\times$) of typical penicillin G acylase crystals. The hexagonal bipyramidal crystals belong to either space group $P6_122$ or $P6_522$ with $a = b = 140.5$ Å and $c = 209.5$ Å. Larger crystals are approximately 0.35 mm in diameter. These particular crystals were grown by the hanging drop/vapor diffusion method from the unblocked P1 preparation (12 mg/mL) in 50 mM K_2HPO_4 , pH 7.5, with 30/45% SAS, 15% (v/v) glycerol, and 0.02% (w/v) sodium azide.

complete to 3.0 Å). Recently, a native crystal was flash cooled to -170°C and a 95% complete data set was collected to 2.6 Å. To date, one successful derivative ($KAuCl_4$) has been identified. Heavy atom sites were determined by difference Patterson and Fourier analysis with HASSP (Terwilliger et al., 1987) and PHASES (Furey & Swaminathan, 1990) in conjunction with direct methods as implemented in SHELXS (Sheldrick, 1990). Heavy atom parameters were refined with PHASES (Furey & Swaminathan, 1990). The calculation of single isomorphous replacement phases with anomalous scattering and solvent flattening is under way, as is the search for additional successful derivatives by cross Fourier analysis.

Discussion

The main difference between the two isoforms of Bro1 penicillin G acylase was conclusively shown to be pyro-Glu formation at the N-terminal Gln of the α -subunit, which is believed to occur during purification and subsequent handling. Spontaneous in vitro conversion of the N-terminal Gln of β -glucanase to pyro-Glu is reported at slightly acidic to neutral pH and room temperature after several days (Yuuki et al., 1989). These conditions are sufficiently similar to those experienced in the course of our purification to explain the partial conversion. β -Glucanase and the acylase α -subunit, both 24-kDa proteins, showed the same pI difference of 0.2 pH units between their respective unblocked and blocked forms (Yuuki et al., 1989). Evidence for in vivo pyro-Glu formation in silkworm chorion proteins is also reported (Regier & Kafatos, 1981). Emergence of the blocked form from the unblocked form after purification, as observed in the P1

preparation, is believed to be the result of repeated freeze/thaw cycles over the span of several years. Other preparations that remained frozen since purification showed no change in the relative amounts of the two isoforms. Therefore, storage of the separated isoforms at -40°C or lower was sufficient to stabilize the enzyme until used for crystallization. It is uncertain whether the second form of the β -subunit represented the gradual increase of another form with, for example, some side-chain modification. It was not reasonable to speculate about the nature of the modification between the two forms of the β -subunit because the ΔpI of 0.07 pH units was the only measured difference.

Our N-terminal protein sequences were in complete agreement with segments of the translated DNA sequence of the propeptide (Ljubijankic et al., 1992) for the 84 residues (46 from the α -subunit and 38 from the β -subunit) that were sequenced. Alignment of the α - and β -subunit sequences from wild-type *P. rettgeri* with the *E. coli* and *K. citrophila* sequences revealed an average homology of 63%, which underscored the advantage of having a *P. rettgeri* sequence for model building. The N-terminus of the α -subunit, however, was inconsistent with the start proposed from the computer-assisted homology alignment of five penicillin acylase amino acid sequences (Konstantinovic et al., 1994). Our work showed that the α -subunit began three residues later, with Gln 24 instead of Ser 21. This shifted the cleavage site of the signal peptide from the proposed Ser-Leu-Ser to Ser-Phe-Ser. Although Ser-Phe-Ser is not as favorable as the Ala-Leu-Ala found in *E. coli* and *K. citrophila* (an ideal cleavage site with Ala in both the -1 and -3 positions), it is reasonable (Von Heijne, 1983, 1986). In *E. coli* signal peptides for periplasmic proteins, Phe and Leu are found at the -2 position with equal frequency (Sjöström et al., 1987). The experimental determination of Ser 228 as the C-terminal residue of the α -subunit confirmed the proposed site of this cleavage (Ljubijankic et al., 1992).

Because the penicillin G acylase used for this work was derived from spontaneously generated mutants with altered substrate specificities (Daumy et al., 1985b), some difference must exist between the wild-type and Bro1 acylase sequences. Changes in substrate specificity of penicillin acylases due to single-residue mutations in the α -subunit are documented (Williams & Zuzel, 1985; Martín et al., 1990). In the work of both Williams (*E. coli*) and Martín (*K. citrophila*), Met 168 was found to play a key role in determining the specificity. The sequence flanking Met 163 in *P. rettgeri* (VGTMANRFS) is identical to the sequence surrounding Met 168 in *E. coli* and *K. citrophila*. Thus, mutation of Met 163 is one possible explanation for the altered Bro1 specificity. Mutation of Met 163 to Asn, Leu, or Ile could account for the difference of 16.9 ± 1.4 Da seen between the observed ESMS mass of the unblocked α -subunit and the molecular weight calculated from the wild-type sequence. All of the above mutations involve the change of one base, except Asn (ATG to AAT or AAC). The change of Met 163 Ile may be more plausible because it involves the transitional mutation of G to A (ATG to ATA), as opposed to the other changes, which involve transversional mutations. If Met 163 was mutated, and this CNBr cleavage site was lost, then the mass of the combined fragment composed of fragments 7 and 8 would have been just above the ESMS 1-kDa cutoff for all possible mutations (Met 163 to any other amino acid). No proof of the combined fragment was seen by ESMS. The combined fragment could have gone undetected, much like fragment 1, which was known to exist and was not

observed. Digestion with another protease might make the mass of the Met 163 fragment more suitable for ESMS analysis. Determination of the sequence variation(s) will wait for the entire Bro1 amino acid sequence and (or) the high-resolution crystal structure.

Crystals tended to grow faster, larger, and more consistently in drops with glycerol. The use of glycerol (10–20%, v/v) to facilitate crystallization supports arguments in favor of its ability to assist in conformational, or intramolecular, stabilization (Sousa & Lafer, 1990). Obtaining highly purified enzyme was crucial to growing diffraction-quality crystals. Only when the sample purities were greater than 96% did crystallization occur, with one exception (crystals were grown from the initial P2 preparation of only 85% purity). However, the ability to crystallize appeared to not be solely due to increased purity. Separation of the two isoforms was also important for crystallization, because no crystals were grown from any sample, regardless of purity, known to contain both isoforms. Also, more convincingly, crystals were grown from the 98% pure P1 preparation and 96% pure P2 preparation, but not from the P1P2 preparation of intermediate purity. It may be that the N-terminal end of the α -subunit is involved in critical contacts that are disrupted by charge or steric heterogeneity. The crystallization results provided strong evidence in favor of the requirement of isoform separation; however, two caveats leave this issue open to debate. The P1 sample used in the pyro-Glu determination was found to contain both isoforms after several freeze/thaw cycles. Because the P1/P2 content of the P1 preparation was not continuously monitored, the later P1 crystallizations were conceivably conducted with mixtures of both isoforms. Also, whether or not pyro-Glu formation occurred in the crystallization drops was not investigated despite the similarity between the conversion conditions reported by Yuuki et al. (1989) and those used in our crystallizations.

Materials and methods

Protein purification

Penicillin G acylase from *P. rettgeri* (strain Bro1) was purified as previously described (Daumy et al., 1985b) but with an additional step of anion exchange chromatography. The protein preparation was loaded onto a Pharmacia FPLC/MonoQ 10/10 column equilibrated in 10 mM K_2HPO_4 , pH 7.5, and eluted with 0.1% 1 M NaCl/min at 4 mL/min. Each fraction was collected for 2 min (8 mL). This treatment resolved the penicillin acylase into two peaks of similar specific activity. Analytical amounts of the isolated peaks (P1 and P2) were characterized at 0.2% 1 M NaCl/min on Pharmacia FPLC/MonoQ 5/5 (1 mL/min) and SMART/MonoQ (100 μ L/min) columns. The protein samples were desalted into 10 mM K_2HPO_4 , pH 7.5, and concentrated with Amicon 10K (green) centrifugal filters and kept frozen at -40 or -70 °C until thawed for crystallization trials.

Purity assessment

Anion exchange chromatography

Peak areas were calculated from analytical FPLC/MonoQ 5/5 elution profiles generated as described above.

CZE

Samples were diluted 1:1 with water and run in 100 mM NaH_2PO_4 , pH 3.5, with 10% (v/v) ethylene glycol. All runs were done at 25 °C and 20 kV with 5-s injections. Eluent protein was monitored by A_{280} . A Beckman P/ACE System 2000 instrument with associated software (version 1.5) was used to collect and plot the data.

IEF/SDS-PAGE (2D) gels

High-resolution 12.5% 2D gels were run according to the protocol recommended by the manufacturer (as described in the Millipore Investigator™ 2D electrophoresis system manual). A total of 5 μ g of protein was loaded per gel. Gels were calibrated in the first dimension with Millipore ampholyte tubes (1 mm $\times$ 18 cm) over the pH range 3–10 and in the second dimension (22 cm) with Bio-Rad Laboratories molecular weight standards from 16 to 241 kDa. Gels were silver stained and dried by standard procedures. Gels used in Figure 3 were scanned on a UMAX UC630 scanner attached to a Macintosh Quadra 700 using Adobe Photoshop (version 2.5). Scanned images were not altered in any way.

Protein and enzyme activity assays

Protein concentration was determined by nomogram (Warburg & Christian, 1947). The related equation derived from the nomogram was used where the protein concentration in mg/mL equaled $1.59(A_{280} - 0.50A_{260})$ corrected for any dilution of the sample in the cuvette. Acylase activity was measured by the rate of enzymatic decomposition of NIPAB (Sigma N-8883) into PAA and ANB (Kutzbach & Rauenbusch, 1974). The rate of formation of the yellow ANB product was monitored by the increase in absorbance at 405 nm. The assay reaction mixture consisted of 400 μ L of 6 mM (1.8 mg/mL) NIPAB in 10 mM K_2HPO_4 , pH 7.5, 590 μ L of 10 mM K_2HPO_4 , pH 7.5, and 10 μ L of the enzyme solution. Phenylacetyl-*p*-nitroaniline synthesized in-house was initially used in place of NIPAB with equal success. A Hewlett-Packard 8452A diode array spectrophotometer was used for both assays.

Crystallization

Multiple factorial searches were used to identify and refine the crystallization conditions (Carter & Carter, 1979; Carter, 1990). Crystals were grown by the hanging drop/vapor diffusion method. Tissue culture trays (Linbro® 76-033-05) were used for the wells. A 2–10- μ L protein drop was pipetted onto an inverted microscope coverslip (22 $\times$ 22 mm), and the coverslip was righted and placed over a well containing 0.5–1.0 mL of the reservoir solution. A vapor-tight seal was formed with immersion oil that could easily be disrupted to harvest crystals or alter the conditions. All coverslips were silanized with SigmaCote® SL-2.

Amino-terminus sequencing

Sequencing of the β -subunit

The α - and β -subunits were separated by SDS-PAGE, electroblotted onto PVDF membrane, and localized on the membrane by Coomassie blue stain. The portion of the PVDF membrane containing each subunit was excised and subjected

to automated Edman degradation. Only the N-terminal sequence of the β -subunit was obtained by the above procedure unless the sample was previously reacted with pyroglutamate aminopeptidase, in which case the N-terminal sequence of the α -subunit beginning with the second residue was also obtained.

Sequencing of the α -subunit

Intact $\alpha\beta$ heterodimer was exchanged into 10 mM Tris-HCl, pH 8.0, over a Pharmacia FPLC/HR 10/10 fast desalting column and concentrated in Filtron 10K (blue) centrifugal filters. Solid GnHCl was added to the exchanged acylase preparation until the GnHCl concentration reached 5 M. The mixture was allowed to stand at room temperature for 1 h. Dissociated subunits were then separated by preparative size-exclusion chromatography (FPLC/Superose 12) equilibrated in 10 mM Tris-HCl, pH 8.0, with 5 M GnHCl. The α -subunit was dialyzed against water, lyophilized, and subjected to automated Edman degradation. The α -subunit used for the CNBr/ESMS analysis was also isolated by this procedure.

Sequencing of the α - and β -subunits simultaneously

The sample buffer was switched to 0.1% acetic acid with a Pharmacia FPLC/HR 10/10 fast desalting column. The two subunits were N-terminal sequenced in parallel by Edman degradation.

Pyro-Glu determination

Intact $\alpha\beta$ heterodimer was transferred into digest buffer (100 mM Na_2HPO_4 , pH 8.0, 0.1 M GnHCl, 5 mM EDTA, 5 mM dithioerythritol, 5% [v/v] glycerol) with a Pharmacia HR 10/10 fast desalting column. Pyroglutamate aminopeptidase (Boehringer Mannheim 1179861) was dissolved in water and added to the sample. A 1/50 peptidase-to-sample weight ratio was used. The digestion mixture was kept at 4 °C for 48 h and 25 °C for 4 h to complete the digestion. The digested sample was concentrated in Filtron 3K (red) centrifugal filters and later Filtron 10K (blue) filters to hasten the process. The α - and β -subunits from the concentrated digest mixture were separated by SDS-PAGE and sequenced as described under sequencing of the β -subunit. The presence of pyro-Glu in the filtrate was detected directly. The filtrate was concentrated from 300 μL to 50 μL by lyophilization overnight. The material did not go completely to dryness because of the presence of glycerol in the digestion buffer. Acid hydrolysis of the filtrate with 500 μL of 6 M HCl and 5 μL 2-mercaptoethanol (1% [v/v]) was performed to reduce any polypeptide fragments to single amino acids. The filtrate tube was purged with nitrogen and kept at 105–110 °C for 24 h. The sample was dissolved in methanol to approximately 5 mg/mL, and 1 μL was injected onto a Hewlett-Packard 5970B GC/MS. The temperature was held at 35 °C for 3 min, ramped from 35 °C to 250 °C at 10 °C/min, and held at 250 °C for 10 min. Commercial pyro-Glu (Sigma P-3634) was used as the standard.

The recipe for the Laemmli buffer (0.5 M Tris-HCl, pH 6.8, 20% [v/v] glycerol, but no SDS) used for the failed attempts to convert Gln to pyro-Glu was taken from Novex literature on Tris-glycine gels. The MilliBlot anode buffer formulation (25 mM Tris, pH 10.4, with 10% [v/v] methanol) used for the failed conversion attempts was taken from the Millipore manual for Immobilon membranes. Samples were placed in Milli-

Blot anode buffer at 37 °C for 1.5 and 14.0 h. Samples were also placed in MilliBlot or Laemmli buffers at 90 °C for 1.5 h.

Molecular weight and α -subunit

C-terminus determination

Molecular weights of the two forms of the α -subunit were measured by HPLC/ESMS. Initial measurements were done by SCIEX API (Biotechnology Research Institute, Montreal, Quebec, Canada) and later duplicated at Pfizer Central Research (Groton, Connecticut) on a Beckman 126 HPLC/Finnigan TSQ700 mass spectrometer. Purified enzyme was exchanged into 50 mM ammonium formate, pH 6.5, with Amicon 10K (green) centrifugal filters and lyophilized, after which the protein was twice dissolved in water and lyophilized. API samples were resuspended in 100 μL of 10% acetic acid, whereas the Pfizer samples were resuspended in 20 μL of 0.1% aqueous trifluoroacetic acid (TFA). Elution off the C18 column was achieved with 0–100% 0.1% TFA in acetonitrile over 45 min.

Identification of the C-terminal residue of the α -subunit first involved the isolation of the α -subunit as described under sequencing of the α -subunit. The α -subunit was concentrated in Filtron 10K (blue) centrifugal filters, rerun over the FPLC/Superose 12 column to remove residual β -subunit, and re-concentrated. The α -subunit was then dialyzed versus 50 mM ammonium formate, pH 6.5, lyophilized, and digested with CNBr in 70% formic acid. A molar ratio of CNBr to Met cleavage sites of 200 was used. The digested sample was lyophilized, resuspended in 20 μL of 0.1% aqueous TFA, and injected into the HPLC/MS for ESMS analysis. Extreme caution was taken with use of CNBr per the guidelines set forth in the Material Safety Data Sheet.

Protein and nucleic acid database searches

The protein and nucleic acid databases GenBank, EMBL, PIR-Protein, and SWISS-PROT were searched with the Genetics Computer Group (GCG) Wisconsin Package (version 8.0) on a Digital Equipment Corporation Alpha 3000/500 running OpenVMS 1.5. Literature searches of the Medline database were conducted through the National Center for Biotechnology Information with the public-domain Nentrez software (version 1.9) on a 486/50 personal computer running Windows 3.1.

X-ray data collection

Room temperature data were collected on a Siemens multiwire area detector (formerly Nicolet X100) mounted on a Rigaku RU200 X-ray generator run at 40 kV/180 mA with $\text{CuK}\alpha$ radiation. A graphite monochromator and 0.5-mm collimator were used. Crystals were mounted in 1-mm glass capillaries and placed at a crystal-to-detector distance of 20 cm. Step sizes of 0.15–0.20° and collection times of 4–6 min per image were used. A helium path reduced air absorption by approximately 30%. The raw images were processed with XGEN/X-GEN (Howard et al., 1987; Howard, 1993). Native crystals lasted up to 14 days under these conditions. Low-temperature data were collected on a Molecular Structure Corporation R-axis IIC equipped with an Oxford Cryo Systems liquid nitrogen device. A step size of 0.5°, collection time of 10 min, and distance of 16 cm were used. The K_2HPO_4 /glycerol storage solution

worked very nicely as the cryosolvent. Raw images were processed with DENZO/SCALEPACK (Otwinowski, 1993).

Acknowledgments

Initial purification and crystallization efforts of Arthur Bourque helped us formulate our strategy. The assistance offered by several people at Pfizer Central Research is very much appreciated, especially Alex McColl for helping with the purification, Joseph Merenda for running the 2D gels, Anthony Lanzetti for sequencing numerous samples, Justin Stroh and Kenneth Rosnack for performing the ESMS analysis, and Gayle Schulte for demonstrating the cryogenic data collection. Discussions with Kieran Geoghegan of Pfizer helped expedite the pyro-Glu determination. Robert Vinopal of the Department of Molecular and Cell Biology at the University of Connecticut provided useful perspectives on the bacteriology. Maria Protá and Kun-Jung Lee assisted with the literature search and sequence comparisons. This work was supported in part by Department of Higher Education grant 00231-17-176. A Connecticut High Technology Scholarship was also awarded to H.E. Klei from 1990 to 1993.

References

- Barbero JL, Buesa JM, Buitrago GG, Méndez E, Pérez-Aranda A, García JL. 1986. Complete nucleotide sequence of the penicillin acylase gene from *Kluyvera citrophila*. *Gene* 49:69–80.
- Bourque AJ. 1988. Isolation, purification, and crystallization studies of strain Bro1 penicillin G acylase from *Proteus rettgeri*. Storrs, Connecticut: Internal report, Department of Molecular and Cell Biology, University of Connecticut.
- Carter CW Jr. 1990. Efficient factorial designs and the analysis of macromolecular crystal growth conditions. *Methods: A Companion to Methods in Enzymology* 1:12–24.
- Carter CW Jr, Carter CW. 1979. Protein crystallization using incomplete factorial experiments. *J Biol Chem* 254:12219–12223.
- Choi KS, Kim JA, Kong HS. 1992. Effects of site-directed mutations on processing and activities of penicillin G acylase from *Escherichia coli* ATCC 11105. *J Bacteriol* 174:6270–6276.
- Daumy GO, Danley D, McColl AS. 1985a. Role of protein subunits in *Proteus rettgeri* penicillin G acylase. *J Bacteriol* 163:1279–1281.
- Daumy GO, Danley D, McColl AS, Apostolakis D, Vinick FJ. 1985b. Experimental evolution of penicillin G acylases from *Escherichia coli* and *Proteus rettgeri*. *J Bacteriol* 163:925–932.
- Daumy GO, Wilder CL, Merenda JM, McColl AS, Geoghegan KF, Otterness IG. 1991. Reduction of biological activity of murine recombinant interleukin-1 β by selective deamidation at asparagine-149. *FEBS* 278:98–102.
- Doolittle RF. 1977. Sequencing peptides and proteins lacking free α -amino groups. In: Needleman SB, ed. *Advanced methods in protein sequence determination*. New York: Springer-Verlag. pp 38–54.
- Frère JM, Nguyen-Distèche M, Coyette J, Joris B. 1992. Mode of action: Interaction with the penicillin binding proteins. In: Page MI, ed. *The chemistry of β -lactams*. New York: Blackie Academic & Professional. pp 148–197.
- Furey W, Swaminathan S. 1990. A program package for the processing and analysis of diffraction data from macromolecules. American Crystallographic Association 1990 Annual Meeting, Abstract PA33.
- Howard AJ. 1993. XGEN and POLYDET: Crystallographic data processing software for the mid-90's. American Crystallographic Association 1993 Annual Meeting, Abstract C001.
- Howard AJ, Gilliland GL, Finzel BC, Poulos TL, Ohlendorf DH, Salemme FR. 1987. The use of an imaging proportional counter in macromolecular crystallography. *J Appl Crystallogr* 20:383–387.
- Huang HT, Seto TA, Shull GM. 1963. Distribution and substrate specificity of benzylpenicillin acylase. *Appl Microbiol* 11:1–6.
- Hunt PD, Tolley SP, Ward RJ, Hill CP, Dodson GG. 1990. Expression, purification, and crystallization of penicillin G acylase from *Escherichia coli* ATCC 11105. *Protein Eng* 3:635–640.
- Konstantinovic M, Marjanovic N, Ljubijankic G, Glišin V. 1994. The penicillin amidase of *Arthrobacter viscous* (ATCC 15294). *Gene* 143:79–83.
- Kutzbach C, Rauenbusch E. 1974. Preparation and general properties of crystalline penicillin acylase from *Escherichia coli* ATCC 11105. *Hoppe-Seyler's Z Physiol Chem* 354:45–53.
- Ljubijankic G, Konstantinovic M, Glišin V. 1992. The primary structure of *Providencia rettgeri* penicillin G amidase gene and its relationship to other Gram negative amidases. *J DNA Sequence Mapping* 3:195–200.
- Martin J, Prieto I, Barbero JL, Pérez-Gil J, Mancheño JM, Arche R. 1990. Thermodynamic profiles of penicillin G hydrolysis catalyzed by wild-type and Met-Ala 168 mutant penicillin acylases from *Kluyvera citrophila*. *Biochim Biophys Acta* 1037:133–139.
- Melius P, Hubbard WL. 1986. Pyroglutamyl N-termini of thermal polyamino acids. *BioSystems* 20:213–217.
- Orlowski M, Meister A. 1971. Enzymology of pyrrolidone carboxylic acid. In: Boyer PD, ed. *The enzymes*, 3rd ed, vol 4. New York: Academic Press. pp 123–151.
- Otwinowski Z. 1993. Oscillation data reduction program. In: Sawyer L, Isaacs N, Bailey S, eds. *Data collection and processing. Publication DL/SC1/R34*. Daresbury, UK: SERC Daresbury Laboratory. pp 56–62.
- Piotraschke E, Nurk A, Galunsky B, Kasche V. 1994. Genetic construction of catalytically active cross-species heterodimer penicillin G amidase. *Bio-technol Lett* 16:119–124.
- Regier JC, Kafatos FC. 1981. In vivo kinetics of pyrrolidonecarboxylic acid formation in selected silkworm chorion proteins. *J Biol Chem* 256:6444–6451.
- Roa A, García JL, Salto F, Cortez E. 1994. Changing the substrate specificity of penicillin G acylase from *Kluyvera citrophila* through selective pressure. *Biochem J* 303:869–876.
- Schultz DE, Spratt BG, Nicholas RA. 1991. Expression and purification of a soluble form of penicillin-binding protein 2 from both penicillin-susceptible and penicillin-resistant *Neisseria gonorrhoeae*. *Protein Expr Purif* 2:339–349.
- Schumacher G, Sizmann D, Haug H, Böck A. 1986. Penicillin acylase from *E. coli*: Unique gene-protein relation. *Nucleic Acids Res* 14:5713–5727.
- Sheldrick GM. 1990. Phase annealing in SHELX-90: Direct methods for larger structures. *Acta Crystallogr A* 46:467–473.
- Sjöström M, Wold S, Wieslander A, Rilfors L. 1987. Signal peptide amino acid sequences in *Escherichia coli* contain information related to final protein localization: A multivariate data analysis. *EMBO J* 6:803–831.
- Slade A, Horrocks JA, Lindsay CD, Dunbar B, Virden R. 1991. Site-directed chemical conversion of serine to cysteine in penicillin acylase from *Escherichia coli* ATCC 11105: Effect on conformational and catalytic activity. *Eur J Biochem* 197:75–80.
- Sousa R, Lafer EM. 1990. The use of glycerol in crystallization of T7 RNA polymerase: Implications for the use of cosolvents in crystallizing flexible proteins. *Methods: A Companion to Methods in Enzymology* 1:50–56.
- Terwilliger TC, Kim SH, Eisenberg D. 1987. Generalized method of determining heavy-atom positions using the difference Patterson function. *Acta Crystallogr A* 43:1–5.
- Von Heijne G. 1983. Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem* 133:17–21.
- Von Heijne G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* 14:4683–4690.
- Waley SG. 1992. β -Lactamase: Mechanism of action. In: Page MI, ed. *The chemistry of β -lactams*. New York: Blackie Academic & Professional. pp 199–228.
- Warburg O, Christian W. 1947. Isolierung und Kristallisation des gærungsferments Enolase. *Biochem Z* 310:384–421.
- Williams JA, Zuzel TJ. 1985. Penicillin G acylase (E.C.3.4.1.11) substrate specificity modification by in vitro mutagenesis. *J Cell Biochem* 9B(Suppl):99.
- Yuuki T, Hidetoshi T, Yabuuchi S. 1989. Purification and some properties of two enzymes from a β -glucanase hyperproducing strain, *Bacillus subtilis* HL-25. *Agric Biol Chem* 53:2341–2346.