The Amino Acid Sequence of *Staphylococcus aureus* Penicillinase

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The amino acid sequence of the penicillinase (penicillin amido- β -lactamhydrolase, EC 3.5.2.6) from *Staphylococcus aureus* strain PC1 was determined. The protein consists of a single polypeptide chain of 257 residues, and the sequence was determined by characterization of tryptic, chymotryptic, peptic and CNBr peptides, with some additional evidence from thermolysin and *S. aureus* proteinase peptides. A mistake in the preliminary report of the sequence is corrected; residues 113–116 are now thought to be -Lys-Lys-Val-Lys- rather than -Lys-Val-Lys-Lys-. Detailed evidence for the amino acid sequence has been deposited as Supplementary Publication SUP 50056 (91 pages) at the British Library (Lending Division), Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies may be obtained on the terms given in *Biochem. J.* (1975) 145, 5.

Many strains of *Staphylococcus aureus* produce an inducible penicillinase (penicillin amido- β -lactamhydrolase, EC 3.5.2.6). In exponentially growing cultures, much of the enzyme is released into the culture medium, and this exoenzyme can readily be purified in large amounts (Richmond, 1963). Enzymes with slightly different chemical and enzymic properties have been found in different isolates of *S. aureus* (Richmond, 1965), and the genes coding for the control and structure of the enzyme have been shown to be carried on an extra-chromosomal plasmid (Novick, 1963). The penicillinase-constitutive strain PC1 (or *pen-c* 1; Novick, 1963) has been used for most biochemical studies of the enzyme, as it produces large amounts of the penicillinase, and a high proportion (about 60%) is excreted into the medium (Richmond, 1963). The remainder of the enzymic activity is firmly bound to insoluble parts of the organism (Saz *et al.*, 1961), and no effective way has been found of solubilizing it.

The penicillinase from *S. aureus* strain PC1 is a basic protein of molecular weight about 30000 (Richmond, 1963). The present paper provides evidence for the amino acid sequence proposed by Ambler & Meadway (1969), and corrects a mistake made in that report. Residues 113–116 were there proposed to be -Lys-Val-Lys-Lys-, but are now thought to be -Lys-Lys-Val-Lys-.

S. aureus strain PC1 penicillinase has proved to be a very interesting protein for the study of denaturation and renaturation kinetics (Robson & Pain, 1973). It has been crystallized, and preliminary information obtained as to the size and symmetry of the unit cell (L. Sawyer, J. Moulton & D. Green, personal communication). Peptides derived from the protein have been used in the development of mass-spectroscopic methods for sequence determination (Morris *et al.*, 1971).

The full publication of the sequence-determination experiments has been delayed while methods for the complete but compact description of such work, using deposited data, have been tried for smaller proteins (Ambler & Wynn, 1973). Further experimental work has also been done with the penicillinase, since its ready availability, ease of proteolytic digestion and highly polar nature make it a very convenient model for testing new methods.

Experimental

Materials

S. aureus proteinase was prepared in this laboratory by Mr. A. Savill, using the procedure of Drapeau *et al.* (1972).^{*} Other enzymes and chromatographic materials were from the sources listed by Ambler & Wynn (1973). Reagents for sequence determination were normal good commercial grades, and were not especially purified. Collidine (used for the accentuation of colours given by amino acids with ninhydrin on paper; Levy & Chung, 1953) was 'collidine fraction' from Midland Yorkshire Tar Distillers, Four Ashes, Staffs., U.K. Colours varied with the grade of collidine used, and the most striking colours were obtained with this especially impure material.

Organism and media

The organism used was *S. aureus* strain PC1, which has been deposited with the National Collection of Industrial Bacteria, Torrey Research

^{*} The strain used was *S. aureus* V8 [Drapeau $\rightarrow$ Wootton (University of Leeds) $\rightarrow$ Savill]. Strain V8 obtained directly from Dr. C. B. Anfinsen (National Institutes of Health, Bethesda, Md., U.S.A.) had a different bacteriophage type and did not produce any proteinase for us.

Station, P.O. Box 31, Aberdeen, U.K., as N.C.I.B. 11195. It is a penicillinase-constitutive strain constructed from the penicillinase-negative strain *S. aureus* N.C.T.C. 8325 by first transducing to it the penicillinase genes from the inducible strain *S. aureus* 524 SC (Rogers, 1953), and then mutagenizing to produce the constitutive strain (Novick, 1963). The organism was grown on a sodium β -glycerophosphate medium (CY; Richmond, 1963) with no inducer present, it having been ensured that the colony from which the inoculum was taken was still making penicillinase. Production has been successfully in 5-litre shake flasks (containing 1 litre of medium), a 20-litre fermenter (Microferm; New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) and a 50-litre fermenter (Fermatic 50 Twin; A.B. Biotec, Stockholm, Sweden). Details of growth conditions have been given by Richmond (1963).

Purification of exopenicillinase

The enzyme was purified by a development of the method of Richmond (1963). The enzyme was adsorbed on to cellulose phosphate from the whole uncentrifuged culture, eluted with 75%-satd. $(\text{NH}_4)_2\text{SO}_4$ solution, and further purified and desalted by gel filtration through Sephadex G-100. Details are given in Supplementary Publication SUP 50056. A more elaborate method is now being used (L. Sawyer & J. Moul, personal communication), as it is no longer being found possible to elute the enzyme from cellulose phosphate so cleanly. It is not known why this apparent change in properties has come about, but an alteration in the properties of commercial cellulose phosphate seems possible.

Starch-gel electrophoresis

Horizontal gels were prepared by the method of Smithies (1959), buffered with 0.02M-potassium hydrogen phthalate (pH4.0). Protein was detected by staining with Naphthalene Black, and penicillinase activity with an iodine-penicillin spray (Dubnau & Pollock, 1965).

Digestion with proteinases

Native *S. aureus* penicillinase is readily digested without prior denaturation by all the proteinases that have been tested. Details of the conditions used for digestion of the protein and for subdigestion of peptides are given in Supplementary Publication SUP 50056.

Separation of 'core' material from soluble peptides

After proteolysis, all tryptic digests and some chymotryptic digests were treated with trichloro-

acetic acid (final concentration 5%, w/v) to precipitate large peptides (Harris & Hindley, 1965). Trichloroacetic acid was largely removed from the supernatant by extraction with diethyl ether.

Fractionation of tryptic 'core' peptides

No completely satisfactory method of fractionating this intractable mixture was discovered. The trichloroacetic acid precipitate was almost entirely soluble in ethanol-formic acid-water (20:1:19, by vol.), and initial fractionation was by gel filtration in this solvent through Sephadex LH-20 or G-50. Final purification of the larger peptides was by further gel filtration (in 50%, v/v, formic acid) or by ion-exchange chromatography on sulphoethyl-Sephadex C-25 in buffers containing 8M-urea. Flow sheets for these fractionations are given in Supplementary Publication SUP 50056.

Separation of peptides by ion-exchange chromatography on sulphoethyl-Sephadex C-25

Because of the high lysine content of the protein, peptic, chymotryptic or thermolysin digestion of the protein formed many medium-sized fragments containing two or more basic residues. Such peptides tend to smear on paper electrophoresis, particularly at pH3.5, and to be recovered by elution in low yield. The middle fractions (V/V_0 1.25-2.0) from the initial Sephadex G-25 gel filtration of these digests were therefore next fractionated by ion-exchange chromatography. Sulphoethyl-Sephadex C-25 was used, as its high capacity allowed peptides to be eluted in relatively concentrated solutions from small columns. Volatile buffer was used, so that subsequent paper separations were not hampered.

Salt-free peptides from the middle fractions of digests of 5-10 μmol of protein were dissolved in 5ml of 0.075M-acetic acid adjusted to pH3.6 with 2M- NH_3 solution, 50 μg of ϵ -N-Dnp-lysine was added, and the mixture applied to a column (5cm $\times$ 1cm diam.) of sulphoethyl-Sephadex C-25 equilibrated with the same pH3.6 buffer. The ϵ -N-Dnp-lysine acted as a visible check on the operation of the column, as it is only just bound to the ion-exchanger under these conditions. Some acidic and neutral peptides are not retarded at all. The column was then eluted with a pH gradient. A two-chambered 'linear' mixer was used, with 120ml of the starting buffer in the first chamber, and a similar volume of 0.4M-acetic acid adjusted to pH8.5 with conc. NH_3 solution in the second chamber. Fractions of volume 2.5ml were collected, and at the end of the gradient 25ml of 2M- NH_3 solution was passed through the column to elute the most basic peptides. Peptides were located by paper electrophoresis of 50 μl portions from each fraction; fractions were

pooled and freeze-dried and then finally purified by paper methods. A representative separation is illustrated in Supplementary Publication SUP 50056.

Peptides were eluted in the approximate order of the number of basic residues contained, those with most basic residues being the most retarded. Peptides with only one basic residue were eluted by about pH4.7.

Fractionation of CNBr peptides

The protein contains only three residues of methionine (Table 1), and one of these is only three residues from the C-terminus (Fig. 1). The three large fragments produced by CNBr cleavage were eventually found to contain 94, 84 and 76 residues (Table 3), too close in size to be satisfactorily separated by gel filtration alone. The CNBr fragments were separated successfully by gel filtration on Sephadex G-75 in 5% (v/v) formic acid followed by ion-exchange chromatography on CM-cellulose at pH5.5 in buffers containing 8M-urea. Details are given in Supplementary Publication SUP 50056.

Methods of amino acid-sequence determination

N-Terminal residues of the protein and of peptides were identified by the dansyl method, and N-terminal sequences by the dansyl-phenyl isothiocyanate method, by using the variations from the standard Gray (1972a,b) methods described by Ambler & Wynn (1973). The maximum number of degradation steps carried out on a peptide was ten.

Peptides were examined by carboxypeptidase as described by Ambler [1972b; methods (a.1), (d) and (e)]. Amide groups were recognized by the electrophoretic mobilities of small peptides, by mobility changes during the successive removal of residues by phenyl isothiocyanate degradation, and by carboxypeptidase release. C-Terminal lysine was removed from some peptides with carboxypeptidase B before phenyl isothiocyanate degradation, so that partial phenyl thiocarbonylation of the ϵ -amino groups should not confuse the electrophoretic-mobility results (Ambler, 1972b). Carboxypeptidase investigation of the whole protein (Table 2) was carried out as described by Ambler (1972a).

Reaction of protein with tetranitromethane

Penicillinase (1 μ mol) was dissolved in 0.2M-Tris-HCl, pH8.7, and 1 μ mol of tetranitromethane (as a 1.5%, v/v, ethanolic solution) added. The reaction mixture was kept at 20°C for 30 min, and the protein then separated from nitroform by gel filtration through Sephadex G-25 into 0.1M-NH₃ solution. The modified protein was then digested successively with trypsin and chymotrypsin, and peptides were isolated by gel filtration through Sephadex G-25

in 5% (v/v) formic acid followed by paper electrophoresis. Peptides containing nitrotyrosines were located by their dark appearance on paper under u.v. light.

Results

Purity of protein

The material used for sequence-determination experiments had a specific activity of about 18 units/ μ g, measured against benzylpenicillin at pH5.9 and 30°C by the Perret (1954) assay; Pollock & Torriani (1953) units (μ mol/h) were used. Protein concentration was measured by acid hydrolysis and amino acid analysis of a sample, and was related to the u.v. absorption of the protein solution. The specific extinction coefficient ($E_{1\text{cm}}^{1\%}$) at 280 nm and pH7 was found to be 6.5, about one-half of the value reported by Richmond (1963). This difference accounts for the specific activity reported here being about one-half of the maximum value attained by Richmond (1963).

When subjected to starch-gel electrophoresis at pH4.0, the protein migrated as one main band, moving rapidly towards the cathode. When the gels were overloaded, a second faint band (staining 2–5% as strongly as the major one) could be seen, with mobility about one-half of that of the main band. The main band had very much penicillinase activity, and there was also considerable activity in the minor band. The assay is very much more sensitive than the protein stain, and there was detectable activity in the whole area of gel swept through by the main band in its migration. When progressively smaller amounts of penicillinase were separated, the enzyme stain first showed the same pattern as before, with the same amount of material in the trail. With lower amounts still, the main band faded out, leaving just the trail; then the trail did not reach as far as the position which would have been reached by the main band, though the portion that did show was still of the same undiminished strength. This phenomenon is interpreted as being due to adsorption of the enzyme on to the starch in such a way as not to inhibit the enzymic activity. The minor band may be a deamidation product, as the protein contains several labile amides. The penicillinase was found to remain at the origin if attempts were made to separate it in starch gels by electrophoresis at pH values greater than 7.

The amino acid composition of the protein preparation, determined by acid hydrolysis and ion-exchange chromatography, is given in Table 1. The values obtained are in very good agreement with those of Richmond (1963) and with those deduced from the proposed amino acid sequence of the protein (Fig. 1). In contradiction to the report of Richmond (1963), no tryptophan was detected in the protein, either by a direct colorimetric method

Table 1. *Amino acid composition of S. aureus penicillinase*

Samples were hydrolysed at 105°C for: (1), 12h; (2), (3) duplicate 24h; (4), 52h; (5), 100h. To minimize the effect of pipetting etc. errors, the recoveries were normalized by multiplying by a factor such that the sums of amounts of all the amino acids except serine, threonine, isoleucine and valine became equal in each analysis. The best value is the average of analyses (2)–(5) for amino acids other than serine, threonine, valine and isoleucine. The 'best values' for serine and threonine were the recoveries extrapolated to zero time of hydrolysis, and for valine and isoleucine extrapolated to infinite time of hydrolysis. Residues/molecule by analysis were calculated from 'best values' by assuming that there were exactly 260 residues in the protein, as the results were calculated before the amino acid sequence (and hence the number of residues expected in total) was known.

	Amount of amino acid recovered (μ mol)					Residues/molecule			
	(1)	(2)	(3)	(4)	(5)	Best value	Analysis	Sequence	Richmond (1963)
Glycine	0.354	0.356	0.355	0.366	0.369	0.361	11.9	12	13
Alanine	0.527	0.544	0.552	0.550	0.544	0.547	18.1	18	20
Valine	0.236	0.354	0.342	0.423	0.452	0.490	16.2	16	14
Leucine	0.542	0.626	0.622	0.648	0.652	0.660	21.8	22	23
Isoleucine	0.291	0.422	0.404	0.490	0.534	0.570	18.8	19	18
Serine	0.544	0.519	0.544	0.522	0.456	0.580	19.2	19	19
Threonine	0.356	0.381	0.396	0.398	0.371	0.410	13.6	13	13
Aspartic acid	1.213	1.291	1.244	1.213	1.194	1.235	40.8	18	42
Asparagine								21	
Glutamic acid	0.509	0.530	0.560	0.560	0.552	0.550	18.2	14	17
Glutamine								4	
Phenylalanine	0.181	0.202	0.200	0.216	0.212	0.207	6.8	7	7
Tyrosine	0.351	0.394	0.384	0.392	0.385	0.389	12.9	13	12
Methionine	0.082	0.092	0.090	0.085	0.093	0.090	3.0	3	2
Proline	0.266	0.277	0.300	0.301	0.299	0.300	9.9	9	10
Lysine	1.085	1.250	1.230	1.269	1.284	1.280	42.3	43	44
Histidine	0.065	0.065	0.061	0.062	0.068	0.064	2.1	2	2
Arginine	0.095	0.113	0.121	0.122	0.125	0.123	4.1	4	4
Total							259.7	257	262*

* Includes two residues of tryptophan, not found to be present at all during the present investigation. See the text.

(Spies & Chambers, 1948) or by quantitative assessment of the u.v. absorption of the protein in acid and alkali (Beaven & Holiday, 1952). No tryptophan-containing peptides were detected at any stage of the sequence determination.

Some preparations of staphylococcal penicillinase (generally, but not always, from strains other than PC1) have been found to yield unexpectedly high amounts of glycine after acid hydrolysis. Such preparations have also given, in good yield after tryptic digestion, a peptide of amino acid composition Gly₅, Ala₂, Glu₁, Lys₁. The significance of the odd amino acid composition, exactly that of the *S. aureus* cell-wall peptide (Ghuysen *et al.*, 1965), was not realized at once. This peptide was subsequently isolated from 'high-glycine' preparations by gel filtration through Sephadex G-75 in 50% (v/v) formic acid, conditions which would not be expected to break normal peptide bonds, and in yields corresponding to about two peptide units per penicillinase molecule. This phenomenon has not been fully investigated, as standard preparations of the enzyme from strain PC1 do not now contain the peptide. Carbohydrate

analyses have not been performed on either 'high-glycine' penicillinases or on the isolated 'cell-wall' peptide. No carbohydrate has been detected in normal preparations of the enzyme from strain PC1.

End groups and polypeptide chain length

Richmond (1963) used the 1-fluoro-2,4-dinitrobenzene method to identify lysine as the sole *N*-terminal residue. This result has been confirmed by the same method in the present investigation, though 'high-glycine' preparations (see above) also yielded Dnp-alanine. No attempt was made to quantify the results, as the penicillinase is a difficult protein to investigate by this method, since there are so many (43) lysine ϵ -amino groups competing with the single α -amino group for the end-group reagent, and the Dnp-protein is very insoluble. Bis-modified lysine is also a difficult compound to handle in either the dinitrophenyl or dansyl methods.

CNBr cleavage of the protein formed the tripeptide Lys-Glu-Phe in good yield (Table 3). As this peptide contained neither homoserine nor its lactone, it was

Table 2. Action of carboxypeptidase A on *S. aureus* penicillinase

Results are expressed as mol of amino acid released/mol of protein. Each digest was of 100nmol of protein for 5h at 37°C. The experimental details are as described by Ambler (1972a). The amino acid columns are arranged in inverse order of the proposed sequence from the C-terminus (Fig. 1). The relative amounts of the two lysine residues released was estimated by interpolation.

Amount of carboxypeptidase (μ g)	Sequence										Impurities and by-products
	-Glu	-Thr	-Ala	-Lys	-Ser	-Val	-Met	-Lys	-Glu	-Phe	
2			0.15							0.94	Nil
10			0.17						0.10	0.98	Nil
20			0.21		0.16	0.20	0.11	0.20	0.19	0.93	0.10 Tyr
50			0.38	0.19	0.40	0.41	0.35	0.42	0.43	0.95	0.10 Tyr
100			0.99	0.85	0.95	0.89	0.80	0.90	0.95	0.96	0.16 Tyr, 0.12 Ile, 0.11 Leu
200	0.19	1.14	1.03	1.04		1.03	0.87	1.16	1.03	0.90	0.16 Tyr, 0.15 Leu, 0.13 Asp, 0.12 Ile, 0.11 Gly

suspected of being derived from the C-terminus of the protein. The dipeptide Glu-Phe was obtained in good yield in tryptic digests, and the whole sequence was deduced (Fig. 1) on the basis that this was the C-terminus. Positive confirmatory evidence was then obtained by carboxypeptidase A digestion of the whole protein (Table 2). The yield of phenylalanine is in good agreement with a polypeptide chain length of about 250 residues.

Richmond (1963) had estimated the molecular weight of the protein to be 29 600 by a sedimentation-equilibrium method. The behaviour of the protein on gel filtration through Sephadex G-100 was consistent with such a size, and the analysis values for amino acids present in small amounts (Table 1; histidine, arginine, methionine and phenylalanine) fitted it well. As the sequence investigation progressed, the predicted numbers of each of these residues were found, and the final value of the molecular weight (28 823), summed from the sequence, shows how extremely good the results obtained by Richmond (1963) were.

Determination of amino acid sequence

The strategy for the amino acid-sequence determination was influenced by the amino acid composition of the protein (Table 1). The high lysine plus arginine content (18%) suggested that, although there would be many peptides to separate in a tryptic digest, their average size would be small, and 'core' problems might not arise. The protein contained neither cysteine nor tryptophan, so no special precautions were necessary to deal with these labile residues. The methionine content (about 1%) was so low that CNBr cleavage was expected to be very useful, though difficulties were anticipated because of the large sizes of fragments. The unfavourable arginine/lysine ratio (1:11) suggested that limited tryptic digestion of lysine-modified protein might be unsatisfactory, as blocking and unblocking

reactions would need to be taken extremely near to completion if the large peptides that should theoretically be formed were to be produced in acceptable yield.

Preliminary experiments showed that the protein [unlike the bacillary penicillinases (Meadway, 1969b; Thatcher, 1975)] was very easy to digest, and that no prior denaturation was necessary. For some digests, the protein was oxidized with performic acid before digestion (Hirs, 1956). This was done to minimize enzymic cleavage at methionine (by chymotrypsin or thermolysin) or to give better yields of methionine peptides by total conversion into the stable sulphone.

Tryptic, chymotryptic and peptic digests of the protein were examined, and as many peptides as possible from each characterized. More than one digest was necessary with each enzyme, because of low yield or non-recovery of desired peptides. Peptides were missed from every digest, either in recognized accidents or because they had properties that made them behave unusually during attempted purification. Especial care was then taken in the repeat experiment (or experiments) to ensure that the same peptide was not lost again. Characterization of the sets of peptides from these three types of digest enabled most of the sequence to be assembled (Fig. 1), but certain ambiguities still remained (Table 5). Characterization of the large peptides formed by CNBr cleavage (Tables 3 and 4) enabled some of these ambiguities to be resolved, and another was solved by the examination of selected peptides from a thermolysin digest of the protein. Long after the final ambiguities had been solved, the protein was examined as a test system for the newly available *S. aureus* proteinase, and the peptides characterized from this digest further confirmed the proposed sequence.

The methods used for the purification of the larger peptides have been described in the Experi-

mental section. After initial batch separation by gel filtration or ion-exchange chromatography (on sulphoethyl-Sephadex C-25), the smaller peptides were fully purified by paper methods, high-voltage electrophoresis at pH 6.5, 3.5, 2 or 9, and paper chromatography (Ambler, 1963; Ambler & Wynn, 1973). Peptides generally required three successive paper purification steps before the analytical criteria given below could be met. The purified peptides were analysed for amino acid composition quantitatively, and terminal groups were determined. Their sequences were determined (as far as the quantity of material allowed) by the dansyl-phenyl isothiocyanate method and by further subdigestion. Yields of peptides varied over a wide range, from over 30% to less than 1%, and did not follow an obvious pattern. Some of the peptic peptides were recovered in very good yield, but many were not, and none was recovered from the region of sequence 17–46 in satisfactory yield (Fig. 1).

The major peptides isolated from each digest, together with peptides isolated after subdigestion of these principal peptides, are shown in Fig. 1. Many more peptides were isolated in low yield, and are described in detail in Supplementary Publication SUP 50056. All peptides isolated except those noted

in Table 5 were completely consistent with the sequence proposed in Fig. 1.

Characterization of large peptide fragments

Large fragments that could not be purified in high yield by paper methods occurred in all the proteinase digests (except that with thermolysin), and formed almost the entire CNBr cleavage mixture. All the sequence could be (and was) examined as small soluble peptides from one or other of the digests, but characterization of the large fragments was attempted to resolve some of the ambiguities (Table 5) and for the sake of completeness.

Both the chymotryptic and the peptic digests contained peptides derived from a very basic region of sequence (residues 104–132; Fig. 1) that were difficult to separate by paper methods. The peptic peptide was obtained pure, but the chymotryptic one was never isolated in an adequately homogeneous state (as is discussed below). Against expectation, the tryptic digests did prove to contain a complex mixture of large non-polar peptides, making up 20–25% of the protein. These fragments were separated from the more tractable

Table 3. *Predicted and observed amino acid compositions of large CNBr fragments from S. aureus penicillinase*

The small fragment X- δ (residues 255–257; Lys-Glu-Phe) was purified by paper methods and fully characterized. (1) and (2) are two possible arrangements of residues (Fig. 1) to make the whole sequence: (1), fragment X- α is residues 1–58 and 88–94, fragment X- β is residues 95–160, 59–87 and 161–178; (2), fragment X- α is residues 1–94 and fragment X- β , residues 95–178. In both cases fragment X- γ is residues 179–254. Δ is the difference between the predicted and observed values for each amino acid. N is the predicted number of residues in each fragment. The observed values have been calculated so as to make the sum of the observed residues equal to the sum of those predicted.

	Fragment X- α				Fragment X- β				Fragment X- γ	
	Predicted	Observed	Observed	Predicted	Predicted	Observed	Observed	Predicted	Predicted	Observed
	(1)	(1)	(2)	(2)	(1)	(1)	(2)	(2)		
Glycine	2	2.4	3.4	3	6	7.2	5.3	5	4	4.2
Alanine	8	6.0	8.7	9	5	5.8	4.3	4	5	5.0
Valine	3	4.2	6.1	6	6	4.8	3.6	3	7	7.1
Leucine	7	6.1	8.8	9	11	12.1	9.0	9	4	4.3
Isoleucine	4	5.6	8.1	8	10	8.7	6.4	6	5	5.1
Serine	6	5.0	7.2	7	6	7.0	5.2	5	7	7.1
Threonine	2	2.2	3.2	3	7	8.1	6.0	6	4	4.2
Aspartic acid	8	9.0	13.0	13	17	15.8	11.8	12	14	14.1
Glutamic acid	6	5.1	7.4	7	7	8.0	5.9	6	4	4.0
Phenylalanine	2	1.5	2.2	2	2	2.8	2.0	2	2	2.0
Tyrosine	4	3.6	5.2	6	6	5.5	4.1	4	3	2.8
Homoserine	1	0.4	0.6	1	1	1.0	0.7	1	1	0.7
Proline	1	1.5	2.2	2	4	3.9	2.9	3	4	4.0
Lysine	9	10.0	14.4	15	22	19.4	14.4	16	11	10.1
Histidine	1	1.4	2.0	2	1	0.3	0.2	0	0	0.0
Arginine	1	0.8	1.2	1	2	2.8	2.0	2	1	1.2
Total	65	64.8	93.7	94	113	113.2	83.8	84	76	75.9
100 $\Sigma(\Delta)$		20	5			14	5			4
N										

Table 4. *Peptides identified in tryptic digests of large CNBr fragments X- α and X- γ*

The peptides were characterized by qualitative amino acid analysis, *N*-terminus and electrophoretic mobility at pH 6.5. The numbers in parentheses refer to positions in the sequence (Fig. 1).

	Peptides obtained pure		Peptides recognized but not fully purified	Peptides expected but not found
Fragment X- α	T59 (1-8)	T73c (58-60)	T83a (56-57)	T58g (10-22)*
	T73a (23-25)	T62f (58-61)	T63f (62-66)	T-core-A (43-55)*
	T77c (26-28)	T511 (67-78)	X α T310 (88-94)	
	T74d (29-34)	T84 (79-82)		
	T75c (35-42)	T67a (83-87)		
Fragment X- γ	X γ T14 (179-182)	T65d (202-211)	T67b (183-189)	T77d (212-220)†
	T77b (190-194)	T57d (238-244)	T87c (235-237)	T-core-B (221-234)*
	T97a (195-197)	X γ T12 (252-254)	T57c (245-251)	
	T77f (198-201)	T914 (256-257)‡		

* 'Core' peptides.

† Probably lost by elution error.

‡ The presence of this peptide shows that the material digested contained some fragment X- γ + δ because of incomplete cleavage of peptide bond 254-255 by CNBr.

part of the digest by precipitation with 5% trichloroacetic acid, but no completely successful method of further fractionating them was discovered. Most of the expected fragments were obtained pure although in low yield. The methods tried are described above, and (in fuller detail) in Supplementary Publication SUP 50056.

The large fragments from CNBr cleavage of the protein were separated by gel filtration followed by ion-exchange chromatography in buffered 8M-urea. The mixtures were much more complex than expected from the methionine content of the protein, but this was apparently not due to cleavage of other peptide bonds. Characterization of the isolated fragments showed that they were all probably charge variants of the three main peptides (X- α , X- β and X- γ ; Fig. 1 and Table 3). The charge variation was presumably due to amide loss and to homoserine-homoserine lactone interconversion. The amino acid compositions of the best-resolved variants of fragments X- α , X- β and X- γ are shown in Table 3. The variants of fragments X- α and X- γ that were judged pure by analytical tryptic peptide 'mapping' were pooled, digested with trypsin and the peptides formed characterized (Table 4). The *N*- and *C*-terminal tryptic peptides were particularly sought after. They were found and analysed, but, because of the small amount of CNBr fragments digested and the complex mixture of tryptic peptides formed, they were not obtained completely pure (see below).

Peptide from maleylated protein

The only peptide characterized from a tryptic digest of maleylated (Butler *et al.*, 1969) penicillinase had the structure

Leu-Lys-Glx-Leu(Gly, Val₂, Thr, Asx₂, Pro, Lys, Arg)

It corresponds to residues 119-131 (Fig. 1), and is the only small peptide expected from cleavage of the molecule at arginine residues only. If the structure shown in Fig. 1 is correct, the other fragments would have lengths of 34, 46, 74 and 80 residues. Trace amounts of a large number of different small peptides were present after maleylation, tryptic digestion and demaleylation, but only the peptide shown above could be characterized.

Experiments to resolve sequence ambiguities

In the preliminary publication (Ambler & Meadway, 1969), the sequence for the region of residues 113-116 was given as

-Lys-Val-Lys-Lys-

When the results were assembled for full publication, it was realized that there was no evidence to prefer this sequence to

-Lys-Lys-Val-Lys-.

Although a tryptic peptide (Val, Lys₂) had been isolated in very low yield (see Supplementary Publication SUP 50056), and had also been identified though not characterized in the subdigest of a peptic peptide, the *N*-terminus had not been determined. Dansylation of the ϵ -amino groups of lysine appears to produce sufficient steric hindrance to slow the hydrolysis of the *N*-peptide bond, particularly if the other residue is valine or isoleucine. The failed dansyl sample was only hydrolysed for the standard 12h, sufficient explanation for the failure.

This sequence ambiguity was resolved by the characterization of the small very basic peptides formed by thermolysin digestion of the protein.

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-Lys-Val-His-Ile-Asn-Lys-Asp-Ile-Val-Ala-Tyr-Ser-Pro-Ile-Leu-Glu-Lys-Tyr-Val-Gly-Lys-Asp-Ile-Thr-Leu-Lys-Ala-Leu-Ile-
61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90

(continued on p. 208)

(continued on p. 210)

(continued on p. 212)

Fig. 1. Evidence for the amino acid sequence of *S. aureus* penicillinase

Peptides derived by digestion with trypsin (T), thermolysin (H) and *S. aureus* proteinase (F) are shown above the sequence, and by digestion with chymotrypsin (C) and pepsin (P) or cleavage with CNBr (X) below the sequence. Principal peptides only are shown except for the *S. aureus* proteinase digest. Peptides from subdigests are labelled with a second capital letter indicating the subdigestion enzyme (S, subtilisin B; B, carboxypeptidase B). MT designates peptides formed by tryptic digestion of maleylated protein or peptides. Full lines indicate quantitative amino acid analyses, substandard if marked *, and particularly bad (see the text) if marked **. Broken lines indicate qualitative analyses. Barred lines indicate minor peptides from the *S. aureus* proteinase digest. Under peptide lines, → indicates end groups and subsequent residues revealed by phenyl isothiocyanate degradation identified by the dansyl method, substandard if shown --. Peptides marked † were examined by carboxypeptidase A digestion. Large circled numbers near the sequence refer to the numbered points in Table 5.

These peptides were isolated by gel filtration through Sephadex G-25 in 5% (v/v) formic acid, in the V/V_0 1.8–2.1 fraction. The complex mixture of small peptides was then separated by ion-exchange chromatography on sulphoethyl-Sephadex C-25. The desired peptides all contained at least two basic residues, and so were retained until after the gradient reached pH 4.8, by which stage all the other peptides had been eluted. The expected peptides were Ile-Lys-Lys (112–114) and Val-Lys-Gln-Arg (115–118), or Val-Lys-Lys-Gln-Arg (114'–118), together with Leu-Asn-Lys-Lys (58–61) and Leu-Ser-Lys-Glu-Asn-Lys-Lys (166–172). All these peptides except Val-Lys-Lys-Gln-Arg were characterized in good yield, showing that the original guess (Ambler & Meadway, 1969) had been wrong.

An extracellular proteinase has been purified from some strains of *S. aureus* (Drapeau *et al.*, 1972), although it is not produced by strain PC1. The proposed specificity, cleavage after glutamic acid and aspartic acid residues, has very great potential use for sequence determination, so some of the proteinase was made and tested on the penicillinase. All the high-yield peptides isolated and characterized (by amino acid analysis and *N*-terminal group) were consistent with the proposed specificity. Under the digestion conditions used (pH 8.5 in ammonium acetate buffer), almost all the bonds adjacent to glutamic acid residues appeared to be hydrolysed, whereas only a very few of the bonds next to aspartic acid residues were cloven. One peptide, partly purified in very low yield, appeared to have been formed by hydrolysis after a tyrosine residue. The peptides isolated are shown in Fig. 1. The expected peptides that were not found would have been of a size or amino acid composition probably to have made them insoluble for the normal purification conditions. Some of the peptides isolated provided confirmatory evidence for weak overlaps (Table 5).

Reaction of the penicillinase with tetranitromethane

The iodine-sensitivity of the enzyme (Richmond, 1963) suggests that tyrosine residues may be involved in the catalytic activity. To investigate this hypothesis, the *S. aureus* penicillinase was treated with tetranitromethane (Sokolovsky *et al.*, 1966). With equimolar amounts of reagent and penicillinase, enzymic activity was decreased to about 50% of normal, but was not decreased below about 10% even with a 20-fold excess of reagent. Penicillinase that had been treated with an equimolar amount of tetranitromethane was digested with trypsin followed by chymotrypsin. The peptides were separated by gel filtration through Sephadex G-25 in 5% (v/v) formic acid and paper electrophoresis. The largest part of the nitrotyrosine was in a peptide of amino acid composition: Asp, 2.0; Glu, 0.4; Ala, 1.0; Val, 0.6;

Ile, 0.7; Leu, 0.2; Tyr, 0.4; 3-nitrotyrosine, 0.4; Gly, 0.12. The electrophoretic mobility at pH 6.5 was -0.73 (relative to Asp = -1.0), and the *N*-terminus was Asx (with a trace of Glx). These results are interpreted as showing that tyrosine-72 reacts most rapidly with tetranitromethane, the peptide isolated being residues 67–72 contaminated with 10% of residues 133–136. The low recovery of the valine and isoleucine was due to the stability of peptide bond 69–70 to 24 h acid hydrolysis. The contaminant could not have been peptide C612a (133–139, containing tyrosine-138 and tyrosine-139) or a peptide of residues 133–138, as these both have much lower electrophoretic mobilities (see Supplementary Publication SUP 50056). The tyrosine and nitrotyrosine forms of peptide residues 67–72 could be separated by electrophoresis at pH 3.5, and were present in nearly equal amounts. No other nitrotyrosine-containing peptides were present in large enough amounts to be characterized.

Peptide nomenclature

Peptides are identified by the system described by Ambler & Wynn (1973). The initial capital letter indicates the method used for the primary degradation of the protein, and other capital letters indicate that the peptide is a subdigestion product. The letters used are: T, trypsin; C, chymotrypsin; H, thermolysin; P, pepsin; S, subtilisin B; F, *S. aureus* proteinase; X, CNBr. MT indicates protein or peptide treated with trypsin while maleylated.

Presentation of sequence evidence

The evidence for the proposed amino acid sequence of *S. aureus* strain PC1 penicillinase is summarized in Fig. 1. This shows the principal peptides isolated from tryptic, chymotryptic, peptic and *S. aureus* proteinase digests of the protein, and selected peptides from a thermolysin digest. The proposed locations of the CNBr peptides are also shown in Fig. 1, and the evidence for the extent of the large CNBr peptides is given in Tables 3 and 4. Peptides formed by secondary digestion are also shown in Fig. 1. Symbols show how much of the sequence of each peptide has been determined by the dansyl-phenyl isothiocyanate method, as well as peptides that were examined with carboxypeptidase. Cases in which the results of dansyl-phenyl isothiocyanate degradation or quantitative amino acid analysis were not considered wholly satisfactory are indicated.

Dansyl-phenyl isothiocyanate degradation results were considered unsatisfactory when more than one dansyl-amino acid was identified as being present in significant amount (subjective visual estimate of more than 20%) at a step of the degradation, or when

Table 5. *Ambiguities, false hypotheses, weak evidence and possible errors in the deduction of the amino acid sequence of S. aureus penicillinase*

Error	Sequence	Comments
1	Overlap 29 -Val-Lys-Phe 29 Phe-Asn-Ser-	Remainder of the Phe residues accounted for in rest of sequence. Sequence similar to that of <i>B. licheniformis</i> protein (Fig. 2).
2	Overlap 35 -Lys-Arg-Phe 35 Phe-Ala-Tyr-	Covered by low-yield peptide P75a (Fig. 1). Remainder of Phe residues accounted for in the rest of the sequence.
3	Overlap 58 -Asn-Lys-Leu 58 Leu-Asn-Lys Asn-Lys-Lys-	Established by composition etc. of fragment X- α (Tables 3 and 4). See also composition of peptide F26d.
4	Overlap 87 -Thr-Leu-Lys 87 Lys-Ala-Leu-	Established by composition etc. of fragment X- α (Tables 3 and 4).
5	Overlap 132 -Val-Arg-Tyr 132 Tyr-Glu-Ile-	Remainder of Tyr residues accounted for in rest of sequence. Close sequence similarity to <i>B. licheniformis</i> protein. See also composition of peptide F37c.
6	Overlap 160 -Asn-Lys-Leu 160 Leu-Ile-Ala-	Established by composition of fragment X- β (Table 3). See also composition of peptide F24g.
7	Very hydrophobic region; sequence and overlap 228-229 -Pro-Ile-Val-Leu-Val-Ile-Phe- 226 227 228 229	The evidence for sequence of residues 226-228 is very thin: (1) action of carboxypeptidase A on peptide P47d shows 0.97 Leu > 0.86 Val > 0.33 Ile (mol/mol) released; (2) specificity of chymotrypsin strongly supports Leu-228. The tryptic peptide that bridges the gap (T-core-B) is insoluble and was isolated in low yield. The hypothesis is strengthened by the composition of fragment X- γ (Table 3).
8	Peptide T83a (56-57; Asn-Lys) Asn-Lys-Leu (peptide C54a) occurring twice (residues 56-58 and 158-160)	With the sequence shown, peptide T83a must come from a pseudo trypsin split, despite quite good yield. The yield of peptide C54a is rather low for a double occurrence. The expected main tryptic peptide (residues 43-57) was not isolated, and is presumed lost in the 'core'. There is fair sequence similarity to the <i>B. licheniformis</i> sequence in this region (Fig. 2), with no postulated insertions or deletions.
9	Very basic region -Lys-Lys-Val-Lys-Gln-Arg-Leu-Lys- 113 120	Sequence wrongly reported by Ambler & Meadway (1969). Evidence for the sequence shown is from the very basic peptides from the thermolysin digest.
10	Hydrophobic chymotrypsin-sensitive region (residues 173-182)	The tryptic peptide (T68d) was insoluble until oxidized, and recovered in low yield. Residue 177 (Leu) was not found in any peptic peptide. Many low-yield chymotryptic peptides were formed. There is close similarity to the <i>B. licheniformis</i> sequence in this region (Fig. 2).
11	Low-yield region -Lys-Ala-Ile-Asn-Ser-Ala-Ile-Leu-Leu-Glu- 42 51	Tryptic peptide insoluble and in low yield; chymotryptic and peptic peptides difficult to purify and in low yield. The only direct evidence for the second leucine is the compositions of peptide T-core-A and fragment X- α , but indirect evidence is from the occurrence of peptide C69 and the known specificity of chymotrypsin.
12	Peptide C210a (see Supplementary Publication SUP 50056): composition Ala ₃ , Leu ₁ , Ile ₁ , Ser ₂ , Thr ₁ , Asp ₄ , Glu ₁ , Met ₁ , Lys ₁ ; <i>N</i> -terminus Ser. No tyrosine, and no <i>O</i> -dansyl-tyrosine observed. Yield very low (0.7%)	Composition corresponds to residues 87-94 plus 97-103. The peptides might possibly combine covalently when a labile amide is lost from peptide C510c (97-103) (see the text). Peptide C27e, isolated in similarly low yield, had a composition corresponding to residues 97-103 plus 197-208, and might have been formed similarly.

Error	Sequence	Comments
13	Anomalous results from 197–201 region -Lys-Val-Ala-Asp-Lys- 197 198 199 200 201 (i) Tryptic digestion of peptide C44a yielded two distinct peptides with compositions Ala, Val, Asp, Lys and <i>N</i> -terminal Lys, with mobilities at pH6.5 of +0.75 (yield 1) and +0.46 (yield 6). (ii) A low-yield tryptic peptide (T63e) was isolated from one digest, with composition Ala ₁ , Val ₁ , Asp ₁ , Lys ₂ and <i>N</i> -terminal Val. Mobility at pH6.5 was +0.46.	No convincing explanation has been thought of. If the <i>N</i> -terminal result for peptide T63e was wrong, the anomalous peptide could have been derived by a pseudo trypsin split at Tyr-196, but there is indirect evidence that a mislabelling error had not been made, as a trace of Dns-Val-Ala (or similar stable peptide) was noted as also present. The sequence in this region is very similar to that of <i>B. licheniformis</i> penicillinase (Fig. 2).
14	Possible amide group on Asp-190	As amide groups from -Asn-Gly- sequences are notoriously labile, all -Asp-Gly- sequences must be regarded as suspect. See the text.
15	Amide group on Asn-65	The evidence is less direct than for the other amides in the protein, depending on quantitative mobility results. See the text.
16	Missing peptide T72c (residues 117–118)	This peptide (Gln-Arg) was not isolated from any of the main tryptic digests, but was found in some minor experiments, although it was not analysed quantitatively and fully characterized. It is probably converted during purification into pyrrolidonecarboxylarginine, which would not have been detected by the ninhydrin reagent used.

for some reason or other (such as accident or shortage of material) the dansyl-amino acid was not positively identified.

The criteria for satisfactory amino acid analyses are: (1) that no impurity should be present in amount larger than 0.20 mol/mol and (2) that, when the relative amounts of amino acids present are calculated on the basis that the average amount is integral, no values should fall outside the limits 0.8–1.2, 1.8–2.2, 2.7–3.3 or 3.7–4.3. Nevertheless, values as low as 0.7 (=1) are considered acceptable for tyrosine, as low yields of this amino acid from pure peptides are often found.

Most of the peptides that did not meet these analytical criteria (marked * on Fig. 1) failed because of slight transgression of the impurity criterion (0.21–0.30 extraneous amino acid) or of the stoichiometry limits (e.g. 0.7 or 1.3 = 1), or because the analysis was satisfactory as far as it went but incomplete. The cases of more serious failure (marked ** on Fig. 1) are discussed individually in Supplementary Publication SUP 50056.

Supplementary Publication

Detailed evidence for the amino acid sequence of the protein has been deposited as Supplementary Publication SUP 50056 with the British Library (Lending Division) for storage on microfiche. This evidence comprises: (1) details of the special methods used in the elucidation of the sequence, and which are not considered to be of sufficient general applicability and interest to justify publication in

the main text; (2) Tables showing the properties of 223 peptides isolated from the protein, including all those shown in Fig. 1; the successive steps used in the purification of each peptide are given, and in suitable cases the values of V/V_0 [elution volume/void volume for gel filtration through Sephadex G-25 in 5% (v/v) formic acid] and electrophoretic mobility at pH6.5. Absolute percentage yields are given for the peptides from tryptic, chymotryptic, peptic and thermolysin digests, and relative yields for peptides from secondary digests. Amino acid analyses are given for all peptides, showing impurities present in amounts greater than 0.1 mol/mol of peptide. (3) Further Tables show the individual sequence evidence for each peptide. This consists of the *N*-terminal-group-analysis results, and the dansyl-phenyl isothiocyanate degradation results and those from carboxypeptidase digestion. Details are given where results are substandard. (4) The evidence for the presence or absence of amide groups on each aspartic acid or glutamic acid residue of the protein is given. (5) A Figure shows all the minor peptides omitted from Fig. 1.

Discussion

Deduction of sequence

The peptides characterized provide highly redundant evidence for most of the sequence shown in Fig. 1. The sequence agrees well with the amino

Fig. 2. Amino acid sequences of penicillinases from *Staphylococcus aureus* PC1 (top) and *Bacillus licheniformis* 749/C (bottom), aligned so as to give maximum matching

Residues that are the same in both sequences are shown in capitals. Regions of weaker sequence evidence (Table 5) are shown; boxed residues are weak overlaps; /, shows peptide bonds that are less well established. The one-letter notation used is that recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochem. J.* (1969) 113, 1-4].

acid composition of the protein deduced from acid hydrolysis (Table 1). Peptides that cover the whole of the proposed sequence have been identified in tryptic, peptic and chymotryptic digests, and after CNBr cleavage (Table 3). Despite this abundance of information, the evidence for some of the sequence is weaker than average, and this is particularly the case for some of the overlaps. These regions of weak evidence are listed in Table 5, and the reasons for preferring the structure shown in Fig. 1 are also given. X-ray crystallographic analysis will eventually show if any of these deductions are false, and may be able to confirm the structure shown in Fig. 1.

Amide groups

A large proportion of published sequence corrections are concerned with incorrectly allocated amide groups. In the present sequence there are 57 sites that could be amidated. There is evidence for the allocation of seven of these sites from carboxypeptidase experiments, although negative evidence (no action by carboxypeptidase at an acidic residue) exists for many more. The evidence for the allocation of all the rest of these sites comes from electrophoretic mobilities at pH 6.5. In all cases except one the amide assignment has been on either the sign (not the quantitative value) of the electrophoretic mobility of a small peptide containing the residue in question (28 cases) or from the study of the change in electrophoretic mobility during phenyl isothiocyanate degradation of a peptide (25 cases). In 11 cases these assignments were made from the mobilities of tryptic peptides which had had their C-terminal lysine residues removed with carboxypeptidase B before phenyl isothiocyanate degradation, so that partial phenylthiocarbamoylation of lysine ϵ -amino groups should not confuse the mobility interpretations.

The one case where the quantitative mobility value has been used in the assignment of an amide group is for residue asparagine-65. The simplest evidence that this residue is asparagine comes from the mobility of peptide T63f (Val-His-Ile-Asn-Lys), which was +0.61 (relative to lysine = +1.0). If residue 65 had been aspartic acid, the net charge on the peptide at pH 6.5 would have been about $\frac{1}{2}+$ (from the partial ionization of the imidazole side chain at this pH), giving a mobility in the range +0.2–+0.4 (Offord, 1966). If the absence of stronger evidence for this assignment had been realized while tryptic peptides were still available, it would have been easy to obtain definitive results, e.g. by thermolysin or carboxypeptidase digestion of peptide T63f. It was not considered justifiable to do a whole protein digest to isolate peptide T63f just to prove this point.

The sequence contained a typically labile Asn-Gly sequence (Ambler, 1963) at positions 163–164. At

least two of the three asparagine residues from residues 99, 102 and 103 were also labile during peptide purification, but not nearly as labile as residue 163. Residue 190 is assigned as aspartic acid, but, as residue 191 is glycine, it must be considered possible that it is really a very labile asparagine. Evidence against this possibility is the homogeneity of peptide T77b (Asp-Gly-Val-Pro-Lys) on electrophoresis at pH 3.5. Deamidation generally produces a mixture of α - and β -aspartyl peptides, which separate well at pH 3.5.

Comparison with sequences of other penicillinases

The amino acid sequence of a *Bacillus licheniformis* penicillinase has been determined (Meadway, 1969a,b; Ambler & Meadway, 1969). This sequence and that of the *S. aureus* protein are shown aligned in Fig. 2. Similarity extends throughout the sequences, and is so great that divergence from a single ancestral gene is the most reasonable explanation. The weaker overlaps in the *S. aureus* sequence (Table 5) are marked in Fig. 2, together with comparably weak overlaps in the *B. licheniformis* sequence (Meadway, 1969b). Some of these weak points are at corresponding places in the two sequences, but at others the sequence similarity is such that evidence from one sequence helps to confirm the arrangement in the other.

The amino acid sequence of the β -lactamase I from *Bacillus cereus* is now almost completely known (Thatcher, 1975), and this also closely resembles the *S. aureus* sequence. It is notable that both of the bacillary penicillinases contain a reactive tyrosine in a similar sequence and corresponding position to tyrosine-72 of *S. aureus* penicillinase, the residue that reacts rapidly with tetranitromethane. A reactive tyrosine in a similar sequence is also present in the penicillinase from *Escherichia coli* strain TEM (Scott, 1973), although the full details of this sequence are not yet known. X-ray crystallographic studies may eventually show if this tyrosine has a catalytic role.

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