Characterization of the Membrane β-Lactamase in *Bacillus cereus* 569/H/9†

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ABSTRACT: The membrane-bound β-lactamase from *Bacillus cereus*, strain 569/H/9, has been purified to apparent homogeneity. Nonionic detergent (0.5% Triton X-100) is required to keep the enzyme (traditionally called γ-penicillinase and now called β-lactamase III) in solution. Antibodies to β-lactamase III have been prepared, and the membrane-bound enzyme is immunochemically distinct from the extracellular enzymes. β-Lactamase III has a molecular weight of 31 500, in contrast to the extracellular enzymes β-lactamase I and β-lactamase II which have molecular weights of 30 000 and 22 000, respectively. The isoelectric point of β-lactamase III is pH 6.8, whereas β-lactamase I and β-lactamase II have isoelectric points about 8.6 and 8.3. The amino acid composition of β-lactamase III differs from those of β-lactamase I and β-lactamase II; however, the difference index between the compositions of β-lactamase I and β-lactamase III (52%) suggests relatedness. β-Lactamase III is inactivated by 6β-bromopenicillanic acid and by the sulfone of 6α-chloropenicillanic acid, and cephalosporins are poorer substrates than penicillins. β-Lactamase III may be a membrane-bound class A β-lactamase.

β-Lactamases are widespread and efficient bacterial enzymes that have attracted considerable attention because of the important part they play in the resistance of pathogens to β-lactam antibiotics. The molecular classification of β-lactamases, based on their primary structures, at present recognizes three classes, named A, B, and C (Ambler, 1980; Jaurin & Grundström, 1981). Class B is distinguished by the requirement of metal ions for activity, whereas class A and class C enzymes, although structurally dissimilar, function by virtue of an active-site serine (Knott-Hunziker et al., 1979, 1980, 1982a,b; Cohen & Pratt, 1980; Fisher et al., 1980, 1981). In Gram-negative bacteria, β-lactamases are commonly located in the periplasmic space, and their efficiency in protecting the bacterial cell from the β-lactam antibiotic has been ascribed to their strategic location (Richmond & Sykes, 1973). The membrane-associated β-lactamase is immunologically indistinguishable from the periplasmic enzyme (Mäntsälä & Suominen, 1981). In Gram-positive bacteria, β-lactamases are found as distinct species both in the culture medium and as bound to the membrane. Nielsen & Lampen (1983a) have suggested that the latter may be regarded as the counterpart of the periplasmic enzyme of Gram-negative bacteria. Moreover, their site may enhance their effectiveness. The first membrane-bound β-lactamase to be recognized, in *Bacillus cereus* 569, was called γ-penicillinase (Pollock, 1956). This organism is exceptional in producing both class A and class B extracellular β-lactamases; these enzymes, β-lactamase I and β-lactamase II, have been much studied (Kuwabara & Abraham, 1967; Abraham & Waley, 1979; Hill et al., 1980). The membrane-bound β-lactamase, now referred to as β-lactamase III, was distinguished from the extracellular enzymes by not combining with antibodies to the extracellular enzymes and by being tightly bound to the protoplast membrane (Pollock, 1956; Sheinin, 1959). While this material has the properties of an integral membrane protein, the loosely cell-bound enzymes obtained from an acetone-dried powder of washed cells (Kuwabara & Abraham, 1969) were indistinguishable from the extracellular enzymes. The use of nonionic detergents, and ion-exchange chromatography, has enabled us to purify β-lactamase III to apparent homogeneity. This sets the scene for the study of the relationship between membrane-bound and extracellular β-lactamases described in the following paper (Nielsen & Lampen, 1983b).

Materials and Methods

B. cereus 569/H/9, a mutant of strain 569/H (Davies et al., 1974), is a constitutive β-lactamase-producing strain. 6β-Bromopenicillanic acid and β-chloropenicillanic acid sulfone were gifts from Professor P. G. Sammes, University of Leeds. β-Lactamase I was prepared as described (Davies et al., 1974; Baldwin et al., 1980).

Enzyme activity was measured at pH 7, 30 °C, in the pH-stat in 0.5 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton X-100; one unit of activity represents the hydrolysis of 1 μmol/min benzylpenicillin.

Protein was estimated by the method of Bradford (1976), with bovine serum albumin as standard and Triton X-100 (0.5%) in the medium.

For amino acid analysis, protein was hydrolyzed with BDH "Aristar" 6 M HCl containing 1 ppm of 2-mercaptoethanol. For comparison with β-lactamase III, β-lactamase I was also hydrolyzed in the presence of Triton X-100. After hydrolysis for 24, 48, or 72 h at 110 °C, amino acids were determined with an LKB Biochrom 4101 analyzer. Tryptophan was estimated after hydrolysis with 4 M methanesulfonic acid for 48 h at 105 °C (penke et al., 1974). The N-terminal amino acid was investigated by the method of Gray (1972), on protein oxidized with performic acid.

Estimation of Molecular Weight and Isoelectric Point. Electrophoresis in 7.5% polyacrylamide gels in the presence of sodium dodecyl sulfate (Weber & Osborn, 1969) was used, with protein markers from Sigma Chemical Co. Isoelectric focusing was performed on LKB Ampholine PAG plates, pH 3.5–9.5, with markers from Pharmacia. Gels were stained to detect enzymic (β-lactamase) activity with hardened (Whatman No. 54) paper dipped in nitrocefin; proteins were detected by staining with Coomassie Brilliant Blue R250. Discontinuous gel electrophoresis was performed on 20% (w/v) polyacrylamide gels as described by Laemmli (1970). Triton

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Table I: Isolation of β-Lactamase III from *B. cereus* 569/H/9[a]

stage of purification	volume (mL)	activity (units/mL)	concn of protein (mg/mL)	sp act. (units/mg)	yield (%)	purifcn (fold)
extract in buffer A	500	80	2	40	100	1
after CM-50	115	200	0.285	700	58	18
after DEAE	60	220	0.1	2200	33	55

[a] Details of the steps are given in the text. Buffer A is 20 mM sodium citrate, pH 6, containing 0.5% Triton X-100. Activity denotes β-lactamase activity; the assay and the unit are described under Materials and Methods.

X-100 was not added during these procedures.

Immunochemical Methods. Antibodies to β-lactamase III (1 mg/mL, in 0.5% Triton X-100, mixed with 1 volume of Freund's complete adjuvant) were raised in New Zealand White rabbits; a total of 2 mg of β-lactamase III was injected intramuscularly, over 3 months. The serum was kept at 56 °C for 30 min. Double immunodiffusion was performed with 5–10 μL of solution (containing antiserum, antibodies, or enzyme) in the wells; incubation was for 24 h at 37 °C (Ouchterlony, 1968). Antibodies to β-lactamase I were those described previously (Baldwin et al., 1980).

Isolation of β-Lactamase III. *B. cereus* 569/H/9 was grown on the 15-L scale (Baldwin et al., 1980). After 8 h at 30 °C the cells were harvested by centrifugation and stored frozen. The cells were than thawed with 300 mL of 20 mM sodium citrate, pH 6, the suspension was centrifuged, and the pellet was dispersed with fresh 300 mL of buffer (20 mM sodium citrate, pH 6). The cycle of freezing, thawing, and centrifugation was repeated until the total β-lactamase activity in the supernatant was less than 1000 units. The pellet was then stirred with 500 mL of 20 mM sodium citrate, pH 6, and 0.5% in Triton X-100 (buffer A) for 36 h at 4 °C and centrifuged at 75000*g* for 90 min and the supernatant was dialyzed against 20 mM sodium citrate, pH 5, containing 0.5% Triton X-100 (buffer B). The precipitated nucleic acids were removed by centrifugation, and the supernatant was fractionated at 4 °C on a column (6 cm diameter × 13 cm long) of Sephadex CM-50, by elution with a gradient of NaCl (0–0.4 M, 1 L) in buffer B (Figure 1, top). Pooled fractions were dialyzed against 20 mM triethanolammonium chloride, pH 7.6, containing 0.5% Triton X-100, and then fractionated on a column (2.5 cm diameter × 9 cm long) of Sephadex A-50 (DEAE) with a gradient of NaCl (0–0.4 M, 500 mL) in the pH 7.6 buffer (Figure 1, bottom). Pooled fractions were dialyzed against buffer B, and the solution was concentrated by ultrafiltration (Table I).

Results and Discussion

Recognition and Preparation of β-Lactamase III. β-Lactamase III was originally recognized by its indifference to antibodies raised against extracellular β-lactamase (Pollock, 1956), and we confirmed that the activity of the extract obtained from washed, disrupted cells with Triton X-100 was unaffected by antibodies to β-lactamase I, although these caused 94% inhibition of β-lactamase I itself. This effect was not diminished by the presence of β-lactamase III. On the other hand, antibodies to β-lactamase III (referred to later) brought about 90% inhibition of β-lactamase III activity.

The preparation of β-lactamase III depends on the extraction of the enzyme from broken cells by a nonionic detergent. After purification by ion-exchange chromatography, the enzyme was obtained in an apparently homogeneous form;

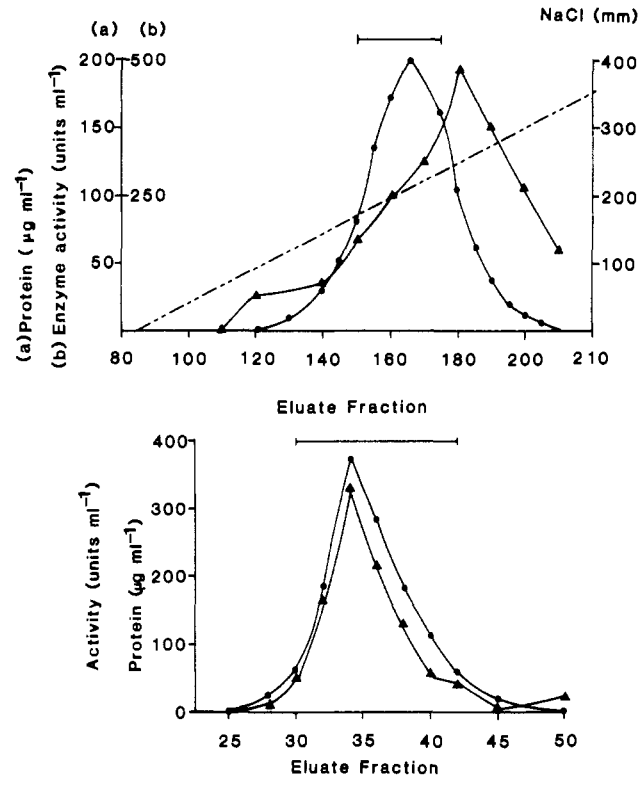

FIGURE 1: Ion-exchange chromatography of β-lactamase III. (Top) The extract, at pH 5, was applied to Sephadex CM-50 and eluted with a gradient of sodium chloride (0–400 mM). β-Lactamase activity (●), concentration of protein (▲), and concentration of sodium chloride (- - -) are shown. (Bottom) Subsequent chromatography on DEAE-Sephadex A-50 at pH 7.6; the enzyme was eluted with a gradient of sodium chloride (0–400 mM). β-Lactamase activity (●) and concentration of protein (▲) are shown.

5–10 mg was obtained from a 15-L batch. Both the yield and the specific activity were about 5-fold greater than those previously obtained (Citri & Kalkstein, 1967). Gel electrophoresis gave one band. Isoelectric focusing gave several close bands, all of which stained for enzymic activity as well as for protein. Ion-exchange chromatography clearly separated β-lactamase III and β-lactamase I, and there was no possibility of confusion with β-lactamase II since assays were carried out in the presence of EDTA.

A Common Control System for β-Lactamase III and β-Lactamase I? A mutant strain of *B. cereus* 5/B, namely, strain 5/B/6, produces no β-lactamase I although it produces a variant of β-lactamase II (Davies et al., 1975). When this strain was used, there was no β-lactamase activity in the Triton X-100 extract. Neither *B. cereus* 569/H/9 nor the mutant strain 569/H/9/2 (Baldwin et al., 1980) gave any appreciable amount of membrane β-lactamase that required Zn(II) for activity.

Molecular Weight and Isoelectric Point of β-Lactamase III. Values of 31 500 and 30 000 for molecular weights of β-lactamase III and β-lactamase I, respectively, were obtained by sodium dodecyl sulfate (SDS) gel electrophoresis. The amino acid sequence of β-lactamase I is not completely known (Thatcher, 1975), but the sequence of the homologous enzyme from *Bacillus lichenformis* (Meadway, 1969) leads to a molecular weight of 29 500. The values obtained by SDS gel electrophoresis are consistent, for β-lactamase I, with values from ultracentrifugation (Hall & Ogston, 1956; Davies et al., 1974).

The isoelectric point of β-lactamase III (main band) was pH 6.8, considerably lower than the values for β-lactamase

Table II: Amino Acid Composition of β-Lactamase III[a]

	composition (nmol/nmol)			
	β-lactamase III		β-lactamase I	
		to nearest integer	present work	Thatcher (1975)
Asp	36.53	37	33	33
Thr	23.47	23	20	21
Ser	16.63	17	13	14
Glu	32.89	33	26	25
Pro	10.2	10	11	10
Gly	14.16	14	19	21
Ala	21.95	22	30	29
Val	12.95	13	13	15
Met	2.98	3	4	4
Ile	12.92	13	20	23
Leu	22.61	23	18	19
Tyr	7.91	8	9	9
Phe	7.54	8	8	7
His	4.73	5	4	4
Lys	27.31	27	24	23
Arg	9.73	10	13	13

[a] Duplicate samples of β-lactamase III were hydrolyzed in 6 M HCl at 110 °C for 24, 48, and 72 h. The values given are based on M_r 29 750 (the average of 30 000 and 31 500, less 1000, an approximate allowance for the lipid moiety. The values for serine and threonine were derived from extrapolations to zero time of hydrolysis and for valine, leucine, and isoleucine from extrapolation to infinite time. The values for β-lactamase I were similarly obtained. No cysteine or cystine (or cysteic acid from oxidized samples) was detected in β-lactamase III, nor was tryptophan after hydrolysis with methanesulfonic acid or with *p*-toluenesulfonic acid.

Table III: Matrix of Percentage Difference in Amino Acid Composition of β-Lactamases and a Carboxypeptidase[a]

	percentage difference index			
	I	III	Cp	Lich
I	0	52	43	41 (44)[b]
III		0	81	52
Cp			0	49
Lich				0

[a] The index $S\Delta n$ is defined as $^1/_2\Sigma(n_{iA} - n_{iB})^2$ where n_{iA} and n_{iB} are the number of residues of the ith type of amino acid in proteins A and B, respectively, each containing approximately N amino acid residues (Cornish-Bowden, 1977, 1980). The value of the percentage difference index (PDI) is then PDI = $100(S\Delta n/N)$. The index (PDI) is an estimator of the number of loci at which the sequences of two proteins differ. The values for the strong test and the weak test of relatedness are 42% and 93%, respectively (Cornish-Bowden, 1980). I, III, Cp, and Lich refer to β-lactamase I, β-lactamase III, the D-alanyl-D-alanine carboxypeptidase from *Bacillus coagulans* (McArthur & Reynolds, 1980), and the β-lactamase from *Bacillus lichenformis* 749/C, respectively. [b] Value in parentheses is from the sequences (Ambler, 1980).

I and β-lactamase II, which are about 8.6 and 8.3, respectively (Baldwin et al., 1980). This difference meant that the first chromatographic step in the purification shed the extracellular enzymes.

Composition of β-Lactamase III. The amino acid composition of β-lactamase III is appreciably different from that of β-lactamase I (Table II). In particular, β-lactamase III has markedly fewer residues of glycine, alanine, and isoleucine. Thus, as inferred earlier (Pollock & Kramer, 1958), β-lactamase III cannot be a precursor of β-lactamase I. Similar reasoning excludes the possibility that β-lactamase III is a precursor of β-lactamase II.

Nevertheless, the values in Table III do suggest that β-lactamase I and β-lactamase III are related. When β-lactamase I and β-lactamase III are compared, the value of the

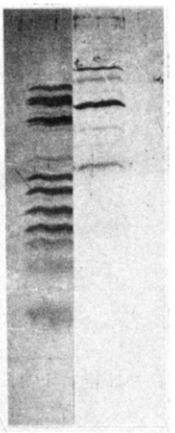

FIGURE 2: Gel electrophoresis of chymotryptic peptides from β-lactamase I and from β-lactamase III. The enzymes (β-lactamase I or β-lactamase III) at 0.5 mg/mL of 125 mM Tris-HCl, pH 6.8, 0.5% in sodium dodecyl sulfate and 10% (v/v) in glycerol, containing 1 ppm of bromophenol blue, were kept at 100 °C for 2 min, and chymotrypsin (1 μg) was added to 40 μg/mL. After digestion at 37 °C for 30 min, 2-mercaptoethanol was added to 10% (v/v) and sodium dodecyl sulfate to 2% (w/v). The mixtures were kept at 100 °C for 2 min, and then 50 μl was applied to the 20% (w/v) (discontinuous) polyacrylamide gel. Electrophoresis (in buffer from 3 g Tris, 14.4 g glycine, and 1 g/L sodium dodecyl sulfate) was for 16 h at 120 V, at pH 8.5. The bands were detected with Coomassie Brilliant Blue R250 (0.1%) in aqueous methanol (50%, v/v) containing acetic acid (10%, v/v). The sample on the left is the digest of 10 μg of β-lactamase I and on the right the digest of 10 μg β-lactamase III. Travel is downward toward the anode at the bottom.

(normalized) difference index is 52%. The value definitely indicates a similarity in the compositions of β-lactamase III and β-lactamase I. Table III also shows some, albeit weak, relatedness between β-lactamase III and the D-alanyl-D-alanine carboxypeptidase of *Bacillus coagulans*, another membrane enzyme, which itself is surprisingly similar in composition to β-lactamase I (McArthur & Reynolds, 1980).

No N-terminal amino acid was detected in β-lactamase III; protein had been oxidized with performic acid before treatment with dansyl chloride. This may be correlated with the hydrophobic moiety reported in the following paper (Nielsen & Lampen, 1983b).

Proteolytic Digestion. Comparison of the structures of β-lactamase III and β-lactamase I was carried out by the method of Cleveland et al. (1977). The proteins were denatured and then treated with chymotrypsin in the presence of SDS. The results were clear-cut: there were few or no bands in common in the SDS gel electrophoresis pattern (Figure 2). An extent of difference of 50% in the amino acid sequences would be expected to lead to very different peptide "maps".

In view of the conclusion that β-lactamase III is not a precursor of β-lactamase I, it is not surprising that attempts to transform the former into the latter were uniformly unsuccessful. In these attempts β-lactamase III was treated with *B. cereus* extracts, or with trypsin, and incubated under various conditions. In no case was there a decrease in the enzymic activity after treatment with antibodies to β-lactamase I.

Immunochemical Comparison of β-Lactamase I and β-Lactamase III. Antibodies to purified β-lactamase III did not affect the enzymic activity of β-lactamase I, but they did, as mentioned above, bring about a decrease in the activity of the β-lactamase III itself. There was no cross-reaction in Ouchterlony plates between antibodies raised toward one enzyme and tested against the other (Figure 3). When purified antibodies were used, there were definite signs of cross-reactivity (Connolly, 1981).

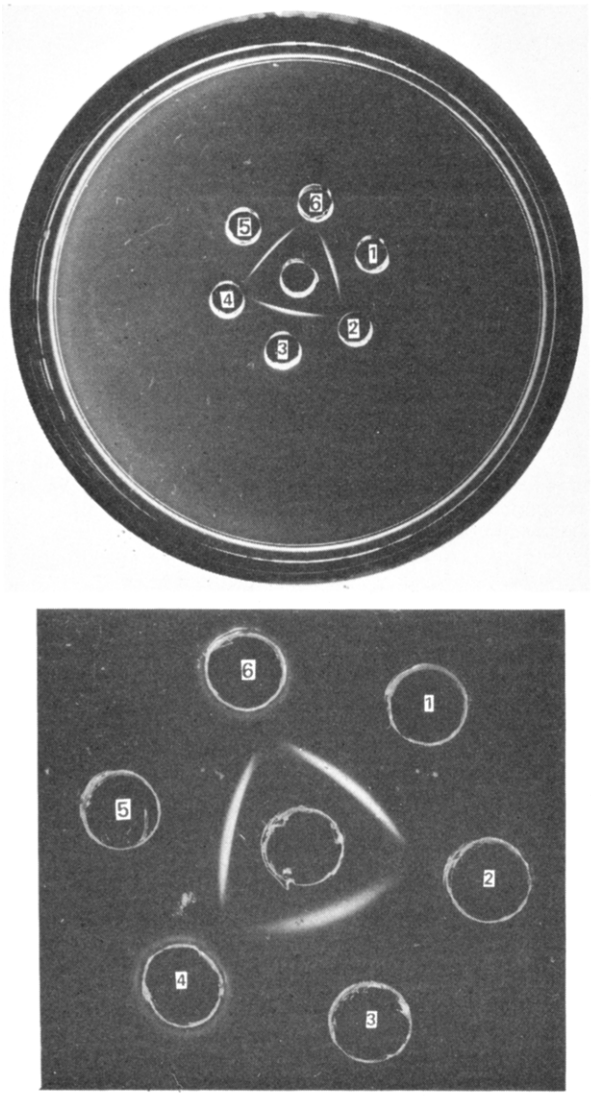

FIGURE 3: Immunochemical comparisons of β-lactamase III and β-lactamase I. (Top) The center well contained antiserum to β-lactamase I. Wells 1, 3, and 5 contained β-lactamase I, and wells 2, 4, and 6 contained β-lactamase III, each at 3 mg/mL. (Bottom) The center well contained antiserum to β-lactamase III. Wells 1, 3, and 5 contained β-lactamase III, and wells 2, 4, and 6 contained β-lactamase I. Each well contained 10 μL of solution, and the plates were incubated for the 16 h at 37 °C.

Activity and Inhibition of β-Lactamase III. The activity of β-lactamase III (and β-lactamase I) toward several penicillins and cephalosporins is shown in Table IV. Both enzymes hydrolyze penicillins in preference to cephalosporins, except for the specially reactive nitrocefin. The specificity of β-lactamase III appears to resemble that of other β-lactamases from Gram-positive bacteria. The absolute values of k_{cat}/K_m for β-lactamase III are somewhat (10–100-fold) lower than those for β-lactamase I, for all but one of the substrates tested (Table IV). Similarly, k_{cat} for benzylpenicillin is about 4-fold greater for β-lactamase I than for β-lactamase III. The exceptional substrate that is hydrolyzed at the same rate by both enzymes is methicillin [see also Citri & Kalkstein (1967)]. Methicillin brings about substrate-induced deactivation of β-lactamase I (Citri et al., 1976; Samuni & Citri, 1979; Kiener & Waley, 1977; Kiener et al., 1980) but behaves as a normal substrate for β-lactamase III. Cloxacillin, however, another substrate of the same class (for β-lactamase I) as methicillin, did cause progressive inhibition of the hydrolysis of benzylpenicillin by β-lactamase III. This is clearly shown by the disparity between progress curves differing by whether enzyme or substrate was added last (Figure 4). Nevertheless, the effect of cloxacillin on β-lactamase III was not as marked as had been observed for β-lactamase I (Kiener et al., 1980), and an approximate value for k_{cat} of the order of 100 min^{-1} for hydrolysis of cloxacillin by β-lactamase III was estimated.

Table IV: Catalytic Activity of β-Lactamase III[a]

	β-lactamase III			
β-lactam	$10^{-4} \cdot k_{cat}$ (min^{-1})	K_m (mM)	log (k_{cat}/K_m) (M^{-1} s^{-1})	β-lactamase I, log (k_{cat}/K_m) (M^{-1} s^{-1})
benzylpenicillin	3.5	0.18	6.5	7.5
ampicillin	2.5	0.5	5.9	7.7
methicillin	0.90	2	4.9	4.9
cephaloridine	0.41	9.5	3.9	5.4
nitrocefin[b]			5.5	7.5

[a] The hydrolyses were measured in the pH-stat at 30 °C at pH 7 in 0.5 M NaCl containing 1 mM EDTA and 0.5% Triton X-100, except for nitrocefin (cephalosporin 87/312) whose hydrolysis was measured spectrophotometrically (O'Callaghan et al., 1972) in sodium phosphate, pH 7: at 500 nm ϵ = 14 mM^{-1} cm^{-1}. The values for β-lactamase I were obtained from parallel experiments, also carried out in the presence of detergent. The values of the kinetic parameters were obtained from direct linear plots (Eisenthal & Cornish-Bowden, 1974). [b] The initial rate was directly proportional to the concentration of substrate, so K_m was too high to estimate, and only the value of k_{cat}/K_m could be obtained.

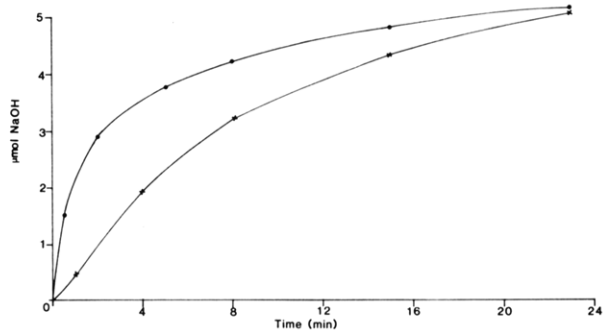

FIGURE 4: Effect of preincubation with cloxacillin on the activity of β-lactamase III. The hydrolysis of benzylpenicillin by β-lactamase III (159 pmol) in 0.5 M NaCl, 1 mM EDTA, and 0.5% Triton X-100 was measured at pH 7, 30 °C, in the pH-stat. In one experiment (●) enzyme was added to the substrate and cloxacillin (10 mM); in the other experiment (×), enzyme and cloxacillin (10 mM) were princubated for 1 min, and then the benzylpenicillin was added. In these experiments the uptake of alkali is due to the hydrolysis of benzylpenicillin, the k_{cat} for cloxacillin being less than 1% of that for benzylpenicillin.

Certain β-lactams act as mechanism-based inhibitors of class A β-lactamases (Newall, 1981; Waley, 1981); one of these, 6-chloropenicillanic acid sulfone (Cartwright & Coulson, 1979), inactivated β-lactamase III. The inactivation was partial but proceeded to completion with a second addition of reagent. Since 2.2 μM enzyme was inactivated to the extent of 63% (in 1 min) by 66 μM reagent, the partition ratio (Waley, 1980) was about 45; i.e., about 1 in 45 turnovers led to inactivation. Another reagent, 6β-bromopenicillanic acid (Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979), also brought about inactivation: the approximate value for the rate constant for inactivation was 0.2 μM^{-1} min^{-1}, one-tenth the value for the inactivation of β-lactamase I (Knott-Hunziker et al., 1980).

Both the activity and the inhibition of β-lactamase III show that it is more similar to class A β-lactamases than to class B or class C enzymes. If the amino acid sequence indicates

that it belongs to class A, then a subclass of membrane-bound class A β-lactamases (perhaps called A_M) may be necessary.

Acknowledgments

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Registry No. β-Lactamase III, 86846-23-3; β-lactamase I, 9001-74-5; benzylpenicillin, 61-33-6; ampicillin, 69-53-4; methicillin, 61-32-5; cephaloridine, 50-59-9; nitrocefin, 41906-86-9; 6β-bromopenicillanic acid, 26631-90-3; 6α-chloropenicillanic acid sulfone, 86953-16-4.

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