Substrate-Specific Inactivation of Staphylococcal Penicillinase

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1. The rate of hydrolysis of methicillin, cloxacillin and quinacillin by staphylococcal extracellular penicillinase decreases progressively with time. 2. The inactivation is prevented but not reversed by benzylpenicillin. 3. The rate of inactivation produced by quinacillin is minimal when the rate of hydrolysis is at a maximum. 4. Under certain conditions, partially inactivated enzyme can be reactivated. 5. Combination of the enzyme with antiserum, while permitting hydrolysis, prevents inactivation. 6. No evidence for a stable enzyme-substrate complex has been found.

The decrease in the rate of hydrolysis of methicillin by staphylococcal exopenicillinase (EC 3.5.2.6) as the reaction proceeds was first noticed by Crompton, Jago, Crawford, Newton & Abraham (1962). Gourevitch, Pursiano & Lein (1962) found similar results with oxacillin and methicillin, with a partially purified cell-bound penicillinase. These authors demonstrated that the product of the hydrolysis, methicilloic acid, did not inhibit the activity of staphylococcal benzylpenicillinase (or methicillinase) and that the methicillin-induced inactivation was prevented by benzylpenicillin. Batchelor, Cameron-Wood, Chain & Rolinson (1963) confirmed that both cell-bound and cell-free penicillinase of *Staphylococcus aureus* were inactivated by methicillin and that this inactivation was prevented but not reversed by benzylpenicillin. They found that cloxacillin was a better inhibitor than methicillin, that the amount of inactivation was not proportional to the amount of cloxacillin hydrolysed and that inactivation was least at pH 6.2, which is the optimum pH for hydrolysis of this substrate.

In a series of papers on *Bacillus licheniformis* penicillinase (see Citri, Garber & Kalkstein, 1964), Citri and co-workers have shown that reaction with methicillin or oxacillin increases the susceptibility of the enzyme to inactivation by iodine, urea or heat. They suggest that there are substrate-induced conformational changes in the enzyme molecule that alter the susceptibility to inactivating agents.

The present investigation was undertaken to study further the substrate-enzyme interactions between the staphylococcal exopenicillinase molecule and various penicillins to see whether evidence for a stable enzyme-substrate complex could be found. The results provide no conclusive evidence for such a complex but suggest that conformational changes may be involved.

MATERIALS AND METHODS

Compounds. The sodium salt of penicillin G (benzylpenicillin) was obtained from Glaxo Laboratories Ltd., Greenford, Middlesex; 6-(2,6-dimethoxybenzamido)penicillanic acid (methicillin) and 6-(3-*o*-chlorophenyl-5-methylisoxazol-4-ylamido)penicillanic acid (cloxacillin) were gifts from Beecham Research Laboratories Ltd., Brockham Park, Betchworth, Surrey; the disodium salt of 3-carboxy-2-quinoxalinylnicillinic acid (quinacillin) was a gift from Boots Pure Drug Co. Ltd., Nottingham.

Media. The organism was grown in 1% CY medium (Novick, 1963), which has the following composition: sodium β -glycerophosphate, 0.12 M; MgSO₄, 1 mM; trace-metal solution, 0.02 ml./l.; yeast extract (Difco), 1.0% (w/v); acid-hydrolysed casein (Difco), 1.0% (w/v); glucose, 0.8% (w/v). The trace-metal solution contained: CuSO₄·5H₂O, 0.5% (w/v); ZnSO₄·7H₂O, 0.5% (w/v); FeSO₄·7H₂O, 0.5% (w/v); MnCl₂·4H₂O, 0.2% (w/v); conc. HCl, 10% (w/v). For solid medium, agar (Difco) (1.5%, w/v) was added to 1% CY medium and the β -glycerophosphate was omitted until after autoclaving.

Organism. *Staphylococcus aureus* 524SC (Rogers, 1953) was the source of the penicillinase used in the experiments. It was maintained by subculture on 1% CY-agar.

Growth of organism and induction of penicillinase. A 50 ml. conical flask containing 10 ml. of 1% CY medium was inoculated from an agar slope of *Staph. aureus* 524SC and incubated with shaking at 35° for 15 hr. This stationary-phase culture was used to inoculate fresh 1% CY medium (50 ml. in a 250 ml. conical flask) at an initial density of 0.05 mg. dry wt. of bacteria/ml. This culture was shaken at 35° for 1 hr. and then methicillin was added to a final concentration of 1.2 μ M and the growth continued for a further 4 hr. (2–3 mg. of bacteria/ml.). Growth and induction were stopped by addition of 8-hydroxyquinoline (Pollock, 1950) to a final concentration of 0.83 mM. The culture was centrifuged at 2000g for 20 min. and the supernatant decanted. This supernatant constituted the crude extracellular penicillinase and had an activity of about 300 penicillinase units/ml. The sedimented cells were washed once in fresh CY medium containing 0.83 mM-8-hydroxyquinoline and resuspended in the same medium.

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This washed-cell suspension was the source of cell-bound penicillinase and was used as such on the same day it was prepared.

Estimation of penicillinase. Penicillinase was estimated iodometrically at pH 5.9 by the method of Perret (1954), as modified by Novick (1962a). One unit of enzyme is defined as the amount that will hydrolyse 1 μ mole of penicillin/hr. at 35° and pH 5.9. The temperature of incubation was 35° rather than 30° used by Pollock & Torriani (1953), so the unit of activity in this paper differs from that more commonly used. The micro-iodometric method of Novick (1962b) was used for determination of initial rates of hydrolysis of the penicillins and the Michaelis constants were calculated from these data.

Purification of extracellular penicillinase. The extracellular enzyme was purified to stage 4 described by Richmond (1963). In brief, the enzyme was adsorbed on cellulose phosphate, eluted with 2M-tris-HCl buffer, pH 7.5, and the eluate dialysed in the presence of CM-cellulose. The cellulose was washed with 0.01M-sodium acetate buffer, pH 5.9, poured into a column, washed again with 0.01M-sodium acetate buffer, pH 5.9, and eluted at pH 5.9 with a gradient made from 200 ml. of 0.02M-sodium acetate in the mixer vessel and 200 ml. of 0.4M-sodium acetate in the concentrated-buffer vessel. Fractions with a specific activity greater than 30 penicillinase units/ μ g. of protein were pooled and constituted the purified enzyme preparation.

Antiserum. Anti-exopenicillinase serum was a gift from Dr M. H. Richmond and was prepared as described by him (Richmond, 1963).

Preparation of methicilloic acid, cloxacilloic acid and quinacilloic acid. The corresponding penicillin was incubated in N-NaOH at 20° for 30 min. The solution was then adjusted to pH 7.0 with N-HCl.

Spectrophotometry. A Unicam model SP.600 spectrophotometer was used with quartz-silica cells of 1 cm. light-path.

RESULTS

The progress curves for hydrolysis of methicillin (17 mM), quinacillin (14 mM) and cloxacillin (15.5 mM) at pH 7.0 and 35° by 40 units of crude extracellular staphylococcal penicillinase/ml. are not linear. The hydrolysis proceeds for less than 30 min. with these concentrations of cloxacillin and quinacillin although less than 2% of each substrate has been hydrolysed. Methicillin hydrolysis continues over the whole 2 hr. of the experiment but is clearly much slower at the end. In the absence of substrate there is little enzyme inactivation under these experimental conditions and hydrolysis of benzylpenicillin (6.7 mM) by 0.4 unit of extracellular penicillinase/ml. is linear, indicating that this substrate does not promote inactivation. The results of similar experiments with cell-bound enzyme show that the progressive reduction in hydrolysis is much less marked for this preparation, but cloxacillin and quinacillin are again more effective inactivators than is methicillin.

Inactivation of benzylpenicillinase. During the

Fig. 1. Inactivation of benzylpenicillinase during the hydrolysis of methicillin, cloxacillin or quinacillin by extracellular penicillinase of *Staph. aureus* 524SC. Penicillinase (50 units/ml.) was incubated at pH 7.0 and 35° with 17 mM-methicillin (○), 15.5 mM-cloxacillin (Δ), 14 mM-quinacillin (□) or buffer alone (●). At intervals 1.0 ml. samples were pipetted into 4.0 ml. of benzylpenicillin (8.4 mM), incubated for 10 min. at 35° and the amounts of benzylpenicilloic acid present estimated iodometrically.

reaction with methicillin, cloxacillin or quinacillin, 1.0 ml. samples were removed and pipetted into 4.0 ml. amounts of benzylpenicillin (8.4 mM) at pH 5.9 and incubated at 35° for 10 min. At the same time 1.0 ml. samples were pipetted into a mixture of 4.0 ml. of benzylpenicillin (8.4 mM) plus 10.0 ml. of 2M-sodium acetate, pH 4.2, containing 4 mM-iodine in 0.16M-potassium iodide to give a blank value. Any benzylpenicilloic acid formed was estimated iodometrically. The results (Fig. 1) show that the benzylpenicillinase activity was very rapidly inactivated by quinacillin and cloxacillin and less rapidly inactivated by methicillin. Under these conditions benzylpenicillin did not reverse the inactivation over the 10 min. incubation period used but did prevent further inactivation.

In a similar experiment in which the extracellular penicillinase was incubated with methicilloic acid (17 mM), quinacilloic acid (14 mM) or cloxacilloic acid (15.5 mM) there was no detectable inactivation of the benzylpenicillinase.

That benzylpenicillin can prevent inactivation was confirmed in an experiment in which extracellular penicillinase was added to three incubation mixtures containing (a) cloxacillin (15.5 mM), (b) cloxacillin (15.5 mM) plus benzylpenicillin (12.5 mM) and (c) cloxacillin (15.5 mM) plus benzylpenicillin (25 mM). The final concentration of enzyme was 50 units/ml. and the assay carried out at pH 5.9 and at 35°. The concentration of benzylpenicillin

used (12.5mm) is in considerable excess of that necessary to saturate the active centre of the enzyme and, further, the concentration of benzylpenicillin used in the assay (6.8mm) would prevent inactivation after a sample had been taken. The results (Fig. 2) show that little or no inactivation of benzylpenicillinase occurs while any benzylpenicillin remains unhydrolysed, but the enzyme is inactivated as soon as all the benzylpenicillin is destroyed. The calculated times for complete

hydrolysis of the benzylpenicillin are 15 min. for (b) and 30 min. for (c).

Kinetics of hydrolysis of penicillins and inactivation of penicillinase. The initial rate of hydrolysis of methicillin, cloxacillin and quinacillin by purified extracellular penicillinase was estimated at many different concentrations of each of the penicillins. A Lineweaver-Burk plot (Lineweaver & Burk, 1934) allowed the calculation of the Michaelis constant and the maximal rate of hydrolysis for each of the substrates (Table 1). Apparent *K* (inactivation) values and maximal rates of inactivation were calculated in a similar manner from the initial rates of inactivation at different penicillin concentrations (Table 1). Cloxacillin and quinacillin have higher affinities for the enzyme than does methicillin, but the maximal rate of hydrolysis of methicillin is higher than for the other two compounds. The apparent *K* (inactivation) values are of the same order as the Michaelis constants for each of the penicillins.

Effect of pH on penicillin hydrolysis and enzyme inactivation. The rate of hydrolysis of methicillin, cloxacillin and benzylpenicillin did not vary much between pH 5.5 and 6.5, whereas, with quinacillin, the pH optimum was 4.8 with a sharp decrease in activity on both acid and alkaline sides. The effect of pH on the initial rate of benzylpenicillin inactivation by quinacillin (1.3mm) was quite different, with a maximum at pH 8.5 and a minimum at pH 4.9. The quinacillin-induced inactivation bore some relationship to the spontaneous rate of inactivation of benzylpenicillinase in that this was highest at the more alkaline pH values.

The effect of pH on the various kinetic values was studied (Table 2). There was little effect of pH on the *K_m* or *K* (inactivation) values, but the maximal rate of hydrolysis and inactivation were inversely related to each other. This suggests that there is a complex of enzyme and quinacillin that under certain conditions (e.g. pH 4.8) breaks down to give active enzyme and quinacilloic acid, but

Fig. 2. Protection of penicillinase against cloxacillin inactivation by benzylpenicillin. Penicillinase (50 units/ml.) was incubated with buffer (○), 15.5mm-cloxacillin (●), 15.5mm-cloxacillin + 12.5mm-benzylpenicillin (△) or 15.5mm-cloxacillin + 25mm-benzylpenicillin (□). At intervals 1.0ml. samples were removed and incubated at 35° for 10 min. with 4.0ml. of benzylpenicillin (8.4mm) and the amounts of penicilloic acid formed estimated iodometrically. At the same times, 1.0ml. samples were incubated for 10 min. at 35° with 4.0ml. of phosphate buffer, pH 5.9 (0.1M), and the amounts of penicilloic acid estimated. This value was subtracted from the experimental value for the corresponding time.

Table 1. *Hydrolysis and inactivation constants for extracellular penicillinase*

The hydrolysis constants were derived from a Lineweaver-Burk plot. Inactivation constants were derived in a similar manner from the effect of substrate concentration on initial rate of inactivation. Both series of experiments were performed at 35° and pH 7.0.

Penicillin	Hydrolysis		Inactivation	
	<i>K_m</i> (mm)	<i>V_{max}</i> (μmoles/hr./50 units of enzyme)	<i>K</i> (inactivation) (mm)	<i>V_{max}</i> (units inactivated/hr.)
Cloxacillin	0.35	0.43	1.1	780
Quinacillin	1.5	0.85	0.2	740
Methicillin	24	1.54	43	320
Benzylpenicillin	0.0025	50	—	—

Table 2. *Effect of pH on hydrolysis and inactivation constants for quinacillin at 35°*

The constants were derived from the effect of substrate concentration on the initial rate of hydrolysis and initial rate of inactivation. $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer (0.1M) was used at pH 5.9 and 7.0. Sodium acetate-acetic acid buffer (0.2M) was used at pH 4.8.

pH	Hydrolysis		Inactivation	
	K_m (mM)	V_{max} ($\mu\text{moles/hr./50 units}$ of enzyme)	K (inactivation) (mM)	V_{max} (units inactivated/hr.)
7.0	1.5	0.85	0.2	740
5.9	1.4	3.2	0.2	240
4.8	1.5	6.7	0.2	90

under other conditions (e.g. pH 8.5) gives rise to either a stable enzyme-substrate complex that is inactive or, relatively slowly, to inactive enzyme and free quinacilloic acid. Conditions were therefore sought under which such a hypothetical inactive enzyme-substrate complex could be reactivated. In addition, experiments were devised to detect directly the presence of quinacillin bound to the enzyme.

Reversibility of the inactivation. Prolonged dialysis of the enzyme, after inactivation with 1.3mM-quinacillin at pH 7.0 at 35° for 30min., against 0.1M-sodium-potassium phosphate buffer, pH 7.0, with or without added benzylpenicillin (6.7mM) did not produce any evidence of reactivation.

Conditions were found in which it was possible to demonstrate reactivation of partially inactivated enzyme. These conditions were critical. They were 0.1M-sodium-potassium phosphate buffer, pH 7.0, 0.1mM-quinacillin and 185 units of exopenicillinase/ml. at 20° (Fig. 3). The benzylpenicillinase activity decreased by 15% in under 10min. and then increased back to the starting level over the next 45min. The point of maximum inactivation corresponds approximately to the point at which all the quinacillin was hydrolysed. Additional enzyme added after 10min. was not inactivated, but additional quinacillin added at this point promoted further enzyme inactivation. If only half as much enzyme was used in the experiment, inactivation occurred but the recovery was very much delayed. With 0.3mM-quinacillin there was an initial rapid inactivation followed by a lower rate of inactivation, but no recovery occurred even after several hours. At higher temperatures (e.g. 25°), under otherwise the same conditions, no recovery was observed.

These experiments demonstrate that the 'new' penicillins and penicillinase can react together to give an inactive form that can be slowly reactivated, but they cannot distinguish between an inactive enzyme-quinacillin complex that is slowly broken

Fig. 3. Reversal of the inactivation of penicillinase. Extracellular penicillinase (185 units/ml.) was incubated in phosphate buffer, pH 7.0 (0.1M), at 20° with: 0.1mM-quinacillin (○) or no quinacillin (△). Benzylpenicillinase was estimated iodometrically at the times indicated. The concentration of quinacillin remaining at these times (□) was calculated by measuring the quinacilloic acid formed micro-iodometrically and subtracting this value from the initial quinacillin concentration.

down and a substrate-induced conformational change in the protein molecule that is slowly reversed. To measure enzyme activity, benzylpenicillin must be used and so this rapidly hydrolysed substrate may be essential for reactivation.

Experiments with antisera. Reaction of staphylococcal exopenicillinase with specific anti-exopenicillinase serum stimulates the maximal rate of hydrolysis up to fourfold (Richmond, 1963). It has been suggested (Pollock, 1964) that this stimulation may be attributed to an altered conformation of the enzyme molecule when combined with the antiserum. If the inactivation produced by

Fig. 4. Protection of penicillinase antiserum against the inactivating effect of penicillinase. Penicillinase (50 units/ml.) + excess of antiserum (○), penicillinase (50 units/ml.) + excess of control serum (△) or penicillinase (200 units/ml.) + excess of control serum (□) were incubated at room temperature for 15 min. and then incubated at 35° at pH 7.0 with 14 mM-quinacillin. The benzylpenicillinase was estimated at a series of time points.

quinacillin were due to some conformational change it might be that reaction of the enzyme with antiserum would stabilize the conformation and so prevent inactivation.

Exopenicillinase (50 units/ml.) was allowed to react with an excess of antiserum (three times the amount required for maximal stimulation of benzylpenicillinase) and then 14 mM-quinacillin was added and the mixture incubated at 35° and pH 7.0. There was no inactivation of the enzyme under these conditions despite the fact that, when an equivalent amount of a rabbit serum containing no penicillinase antibodies was substituted for the antiserum, either 50 units of enzyme/ml. or 200 units of enzyme/ml. were rapidly inhibited under otherwise identical conditions (Fig. 4). On the other hand, exopenicillinase that had been inactivated by quinacillin was not reactivated by the addition of excess of antiserum. Thus antiserum protects the enzyme from the quinacillin-induced inactivation but cannot reverse such inactivation. Reaction with antiserum did not increase the ability of the enzyme to hydrolyse quinacillin under the same conditions that stimulated the ability of the enzyme to hydrolyse benzylpenicillin 3.6-fold.

Exopenicillinase was not inactivated by 15.5 mM-cloxacillin or by 17 mM-methicillin after reaction with antiserum.

Effect of sodium chloride. When the exopenicillinase was treated with 14 mM-quinacillin at pH 7.0 and 35° in the presence of 1.5 M-sodium chloride, no inactivation occurred. Addition of

Fig. 5. Absorption spectra in 0.5 M-NaCl of 0.028 mM-quinacillin (—), purified penicillinase (·····), supposed quinacillin-penicillinase complex (----) and quinacillin-penicillinase complex read against purified penicillinase of equivalent extinction at 278 mμ (— · — ·).

sodium chloride to a final concentration of 1.5 M at any time during the inactivation of exopenicillinase by 14 mM-quinacillin very rapidly prevented further inactivation without promoting reactivation.

Binding of quinacillin to the enzyme. Quinacillin has a side chain that absorbs light with molar extinction coefficients of 32 100 at 242 mμ and 7280 at 326 mμ (Richards, Housley & Spooner, 1963). Purified exopenicillinase has a very low extinction at 326 mμ, so that the presence of quinacillin bound to the enzyme molecule might be detected from the spectrum of the presumed enzyme-quinacillin complex. Theoretically, 0.1 μmole of quinacillin bound to 0.1 μmole of enzyme (approx. 100 000 units) in a volume of 1.0 ml. should have an extinction at 326 mμ of 0.728.

A 5.0 mg. sample of purified exopenicillinase (specific activity 30 units of enzyme/μg. of protein) was treated at 35° with 4 μmoles of quinacillin in 1.5 ml. of 0.1 M-sodium-potassium phosphate buffer, pH 7.0, for 60 min. The quinacillin was added in 0.4 μmole quantities at 6 min. intervals, a procedure that increased the amount of inactivation. The final exopenicillinase activity was 43% of the starting activity. A 1.2 ml. portion of the reaction mixture was layered on top of a column (1.3 cm. × 140 cm.) of Sephadex G-100 and eluted with 0.5 M-sodium chloride at 4°. Fractions (5.0 ml.) were collected and monitored for their extinction at 280 mμ and 326 mμ. Two peaks were separated. The first corresponded to the position expected for the purified enzyme whereas the second corresponded to the position of quinacillin in 0.5 M-sodium chloride. Fig. 5 illustrates the spectrum of one of the fractions of the first peak read against 0.5 M-sodium chloride or against a solution of

purified extracellular penicillinase adjusted to the same extinction at 278m μ . There was no evidence for material absorbing significantly at 326m μ in any of the fractions from the first peak from the Sephadex column.

DISCUSSION

The evidence presented in this paper suggests that staphylococcal penicillinase, like the penicillinases from *Bacillus licheniformis* (Citri & Zyk, 1965), can undergo conformational changes that affect its enzymic activity. Interaction with the 'new' penicillins produces a form of enzyme that is no longer able to react with most substrates, although the partially inactivated enzyme may regain its activity under appropriate conditions. The fact that reactivation is possible at all argues that the initial inhibitory changes do not involve the hydrolysis of any covalent bonds in the enzyme.

The similarity of the values for K_m and K (inactivation) for the various penicillins, and the ability of benzylpenicillin to protect the enzyme from inactivation, suggests that there is only one site of enzyme-substrate interaction. Further, the effect of pH on the kinetics of interaction and hydrolysis of quinacillin suggest that the attachment of this compound to the enzyme is unaffected over a wide range of pH values, yet the rate of hydrolysis of the compound and its efficiency as an enzyme inhibitor are markedly affected. It seems possible that at pH 4.8 the enzyme-quinacillin complex breaks down to give free quinacilloic acid and active enzyme, whereas at pH 8.5 an inactive conformation of the enzyme is produced.

The cell-bound staphylococcal penicillinase has properties very similar to the purified extracellular enzyme (Richmond, 1963), and there is no reason to anticipate that the two types of enzyme have a very different amino acid composition although the former enzyme has never been purified. Yet the cell-bound enzyme is very much less susceptible to inactivation by the 'new' penicillins. All preparations of the cell-bound enzyme yet made seem to be particulate in nature, as though the enzyme was sticking to, or held in, comminuted bacterial cytoplasmic membrane (Richmond, 1963). It is probable that the attachment of the enzyme to these pieces of membrane limits the range of conformational changes possible, and thus the degree of inactivation, when the cell-bound enzyme is

treated with the 'new' penicillins. In a similar way, antiserum might be expected to impose a rigidity on the penicillinase molecule and thus explain the protection antiserum affords against inactivating conformational changes.

The inactivation of penicillinase by the 'new' penicillins, but not by benzylpenicillin, raises the question whether the inactive form of the enzyme has a penicillin residue bound to its active centre. The experiments described above make this possibility unlikely since the maximum absorption that could possibly be due to bound quinacillin in the experiments described was equivalent to less than 0.05 mole of quinacillin/mole of enzyme. It is, of course, possible that the quinacillin may be displaced during the purification; however, there is no recovery of enzyme activity during the separation on Sephadex and it seems most likely that the conformational changes caused by the 'new' penicillins are induced without the function of a stable enzyme-inhibitor complex.

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