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The Active Site of Penicillinase from *Staphylococcus aureus* PC1

ISOLATION OF A SPECIFIC COVALENT COMPLEX WITH THE SUBSTRATE QUINACILLIN

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1. The penicillinase-catalysed hydrolysis of quinacillin was quenched by addition of 5M-guanidinium chloride or 1% (w/v) sodium dodecyl sulphate, and the quenched reaction mixture was dialysed exhaustively against solutions of the denaturant. 2. Irreversibly bound quinacillin was shown by titration with $HgCl_2$ to be covalently attached to the protein by the β-lactam carboxyl group. 3. The derivative was found to be stable over the pH range 3.5–8.5. 4. Chymotryptic hydrolysis of the product and subsequent fractionation showed that quinacillin was bound to one or possibly two peptides.

Penicillinases (EC 3.5.2.6) with various structural properties from various organisms possess the common ability to hydrolyse the β-lactam bond in a variety of penicillins. The architecture of the active site is thus of considerable interest. So far only tyrosine has been implicated as a possible active site group in *Staphylococcus aureus* penicillinase (Ambler & Meadway, 1969). It has been suggested that penicillin is involved in an acylation reaction with a cell wall transpeptidase (Tipper & Strominger, 1965), and it would be reasonable to suggest that the β-lactamase activity proceeds via an acylation step. On this basis an attempt has been made to isolate such a covalent intermediate by quenching the hydrolysis reaction with a denaturing agent which destroys catalytic activity without affecting a possible covalent enzyme–substrate complex. The high absorption of u.v. light by quinacillin made it a suitable substrate. The kinetics of denaturation of *Staphylococcus* penicillinase by guanidinium chloride have also been studied (Robson, 1971) and shown to be very fast at high concentrations, so this denaturant was chosen to quench the enzymic reaction. Sodium dodecyl sulphate, which also denatures penicillin, was used as an alternative quenching reagent.

The results indicate that a stable covalent complex is formed between quinacillin and penicillinase in a molar ratio approaching 1:1.

Materials and Methods

Penicillinase from *S. aureus* PC1 (Richmond, 1963) was purified from partially pure material prepared by the Microbiological Research Establishment, Porton Down, by the method of Robson (1971) who showed preparations to be homogeneous by polyacrylamide-gel electrophoresis at pH 7.1 in either sodium phosphate buffer or in sodium dodecyl sulphate. Preparations had a specific activity of 285 μmol/min per mg. The molecular weight is 28800. It was assayed by following the change in E_{240} of 1.4mM-benzyl penicillin in phosphate–EDTA buffer at 25°C. Specific activity was 285 μmol/min per mg. Benzyl penicillin was a gift from Beecham Research Laboratories, Brockham Park, Surrey, U.K., and was recrystallized three times from methanol–diethyl ether. Quinacillin (3-carboxy-2-quinoxalinyl penicillanic acid; sodium salt) was a gift from Boots Pure Drug Company, Nottingham, U.K.

For quenching, guanidinium chloride was prepared from AnalaR guanidinium carbonate (Anson, 1941); for dialysis, 7M-guanidinium chloride, reagent-grade, was purified by shaking repeatedly with decolorizing charcoal to remove u.v.-absorbing material. Sodium dodecyl sulphate, reagent-grade, and guanidinium chloride, reagent-grade, were from BDH, Poole, Dorset, U.K. Other reagents were AnalaR, when obtainable.

Phosphate–EDTA buffer, pH 7.0, contained 61mM-Na_2HPO_4, 39mM-KH_2PO_4 and 1mM-EDTA (disodium salt). Ammonium acetate buffers were prepared by diluting the appropriate volume of acetic acid to approximately three-quarters of the final volume, adjusting it to the required pH with 35% NH_3 solution and then making it up to the final volume. Concentrations shown are of total acetic acid.

Quinacillin was detected by its absorption at 326nm where the extinction coefficient, ε, was 7280 litre·mol^{-1}·cm^{-1} (Richards *et al.*, 1963). Quinacilloyl groups were estimated from the increase in extinction at 282nm on the addition of $HgCl_2$, as described by Parker *et al.* (1962) except that $HgCl_2$ was added in 5 μl portions, and the E_{282} was allowed to stabilize between additions. The titration was continued until

E_{282} began to decrease. The concentration of quinacilloyl groups was calculated by assuming that ε_{282} was 2.13×10^4, as for penicillin G (Parker *et al.*, 1962). Under the conditions used, the stable increases in E_{282} caused by free quinacillin and by quinacilloic acid were respectively 0.015 and 0.05 times that expected for quinacilloyl groups from the low stability of penamaldates of free penicilloic acids (Schneider & de Weck, 1966*a*). Penicillinase was estimated from its absorption at 276.5nm ($E^{1\%}_{1cm} = 6.87$; R. H. Pain & M. Gambie, unpublished work). Values of $E_{276.5}$ were corrected for any contribution from quinacillin by using a value for $E_{326}/E_{276.5}$ of 2.10 for quinacillin. Quinacillin and protein concentrations were measured in 1cm light-path semi-micro cells (1ml) with the diffusate as blank. Measurements were made at 30°C for solutions containing sodium dodecyl sulphate.

To isolate peptides, 100mg of quinacilloyl penicillinase was digested with 1.5mg of chymotrypsin for 3h at 37°C, as described by Ambler (1963), and then freeze-dried. The digest was dissolved in the minimum volume of 0.05M-ammonium acetate buffer, pH5.0, and applied to a column (1.5cm×80cm) of Sephadex G-25 (Superfine) which had been expanded in 0.1M-NH_3 before being equilibrated with 0.05M-ammonium acetate buffer, pH5.0 (Ambler, 1963). Fractions that absorbed at 326nm were pooled and freeze-dried. This material was dissolved in approx. 2.5ml of 75mM-ammonium acetate buffer, pH3.6, and applied to a column (1.5cm×5cm) of sulphopropyl-Sephadex (C-25) equilibrated with the same buffer. After 10ml in 1ml fractions had been collected elution was continued with a linear gradient of 100ml each of the starting buffer and 0.2M-ammonium acetate buffer, pH8.5. These conditions were the same as those used with sulphoethyl-Sephadex by Ambler & Meadway (1969). Fractions containing quinacillin were pooled and freeze-dried.

High-voltage electrophoresis was carried out on Whatman 3MM paper on a horizontal cooled plate at pH6.5 [1% (v/v) acetic acid–10% (v/v) pyridine] with a potential gradient of 40V/cm.

Results and Discussion

Penicillinase (1.3mg/ml) was incubated with 40mM-quinacillin in phosphate–EDTA buffer at 25°C (see Fig. 1 for typical progress curves). Solid guanidinium chloride or 10% (w/v) sodium dodecyl sulphate in phosphate–EDTA buffer, pH7.0, was added to a final concentration of 5M and 1% respectively at times corresponding to partial and to complete

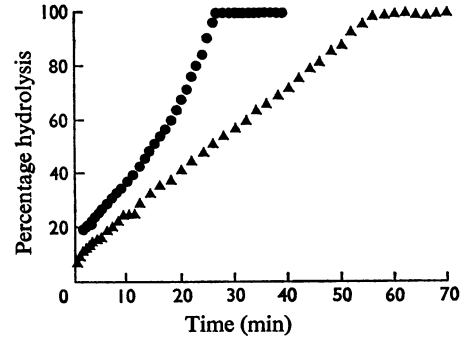


Fig. 1. *Typical progress curves for penicillinase-catalysed quinacillin hydrolysis*

Quinacillin hydrolysis was followed by change in optical rotation at 400nm in phosphate–EDTA buffer, pH7.0, at 25°C (R. Virden & R. H. Pain, unpublished work). Quinacillin and enzyme concentrations were respectively: ●, 40mM and 1.32mg/ml; ▲, 3.6mM and 132μg/ml. At the higher concentration of quinacillin and enzyme the accelerated rate observed after 10min is associated with a fall in pH due to inadequate buffering.

Table 1. *Extent of firm binding of quinacillin after quenching mixtures of enzyme and substrate*

Enzyme and quinacillin were incubated and the reaction was quenched at the times shown, as described in the Results and Discussion section. For samples shown as being quenched at 0min, denaturant was added to the enzyme before addition of quinacillin. After dialysis to remove free quinacillin, samples were taken for estimation of protein and bound quinacillin as described in the Materials and Methods section. Where results were averaged, the number of determinations is given in parentheses.

Method	Time before quenching (min)	Molar ratio of bound quinacillin/protein	
		Spectrum	$HgCl_2$ titration
Guanidinium chloride quenching and dialysis	0	0	0.03
	5	0.77	0.70
Guanidinium chloride quenching (dialysis against 0.1M-ammonium acetate buffer, pH8.5)	10	0.59 (3)	0.34 (3)
Sodium dodecyl sulphate quenching and dialysis	0	0.13	0.02
	5	0.70 (2)	0.47 (2)
	60	0.14	0.06

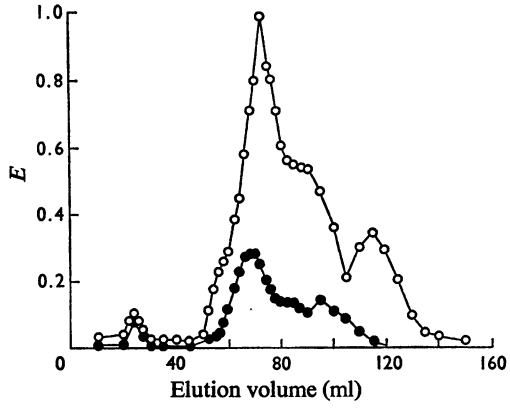


Fig. 2. *Fractionation of a chymotryptic digest of quinacilloyl penicillinase on Sephadex G-25*

A mixture of quinacillin and penicillinase, quenched with guanidinium chloride and dialysed against 0.1 M-ammonium acetate buffer, pH 8.5, as described in the Results and Discussion section was digested with chymotrypsin and applied to a column of Sephadex G-25 as described in the Materials and Methods section. Fractions of volume 1 ml were collected; ○, E_{280} corrected for the contribution of any quinacillin; ●, E_{326}.

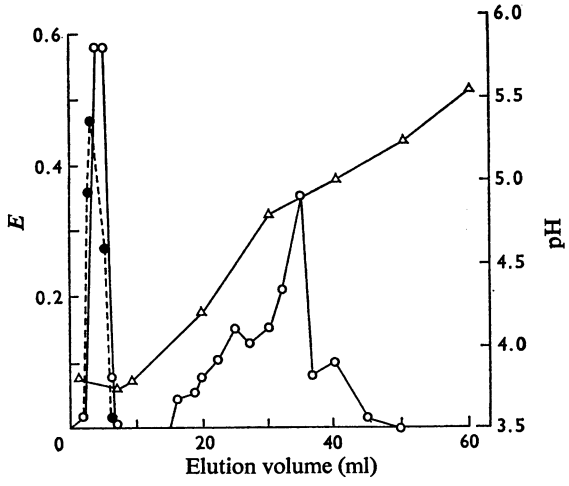


Fig. 3. *Fractionation of quinacillin-containing chymotryptic peptides on sulphopropyl-Sephadex*

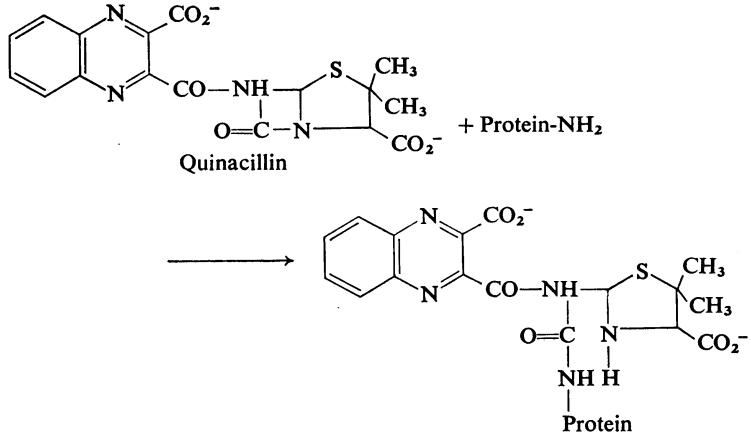
A portion (50%) of fraction CII from the separation shown in Fig. 1 was fractionated on a sulphopropyl-Sephadex column as described in the Materials and Methods section; 1 ml fractions were collected. ●, E_{326}; ○, E_{280}, corrected for the contribution of any quinacillin; △, pH. The peak of E_{326} corresponded to a recovery of 120% of that loaded.

hydrolysis of the quinacillin under these conditions. Samples containing guanidinium chloride were then stored in ice. Those containing detergent were incubated at 25°C for 20 min. It was found that incubation of penicillinase in phosphate–EDTA buffer containing 1% sodium dodecyl sulphate resulted in complete loss of activity after 9.5 min. Samples shown as being quenched at time zero (Table 1) were treated with denaturant before addition of quinacillin. After being quenched, samples were dialysed against solutions in phosphate–EDTA buffer, pH 7.0, of the corresponding denaturant. A total of 13 changes of 25 ml of guanidinium chloride solution at 4°C and five changes of 500 ml of detergent solution at room temperature (20–25°C) were used respectively, until E_{326} of the final diffusate, measured after a minimum 24 h dialysis, was zero.

Significant retention of quinacillin occurred in dialysed solutions of enzyme that had been quenched during enzyme-catalysed hydrolysis of quinacillin, the amount of quinacillin retained being close to 1 mol/mol of protein (Table 1). Less than 0.14 mol was retained when quenching preceded mixing of enzyme with quinacillin or when quinacillin hydrolysis was allowed to go to completion (60 min) before quenching. These controls indicate that non-specific formation of quinacilloyl derivatives of the enzyme was small. In line with this, incubation of quinacillin with lysozyme, another basic protein, under the same conditions yielded no detectable binding of quinacillin. Difference spectra calculated from measurements before and after addition of $HgCl_2$ gave a peak with $\lambda_{max.} = 280$–290 nm, as expected for a penamaldate (Woodward *et al.*, 1949).

For peptide studies, enzyme–quinacillin mixtures quenched with 5 M-guanidinium chloride were dialysed against 0.1 M-ammonium acetate buffer, pH 8.5; recovery of bound quinacillin was around 0.6 mol/mol of enzyme (Table 1). To test the stability of the derivative in the buffers used to isolate peptides, samples (approx. 1.5 mg/ml) were dialysed for 3 days at 4°C in 0.1 M-ammonium acetate buffer at various pH values between 8.5 and 3.5, and then for a further 1 day against 0.1 M-ammonium acetate buffer, pH 8.5. No loss of bound quinacillin was detected.

Fig. 2 shows the elution pattern obtained on Sephadex G-25 with a chymotryptic digest of the quinacilloyl enzyme. The major peak of material, CII (fractions 57–80), emerged together with peptides of molecular weight 1000–2000 (Ambler & Meadway, 1969), which were neutral and basic on high-voltage electrophoresis at pH 6.5. Fractions 81–105 were pooled as CIII. The recoveries of E_{326} in fractions CII and CIII were 26 and 23% respectively of that present in the digest before freeze-drying. The pooled quinacillin-containing fraction, CII, was unretarded on



Scheme 1. *Possible nature of covalent attachment of quinacillin to penicillinase*

sulphopropyl-Sephadex at pH 3.6 (Fig. 3). An elution profile essentially identical with that shown in Fig. 3 was obtained for fraction CIII. The possibility that the unretarded material derived from fraction CII contained free quinacillin was investigated by dissolving one-third of it after freeze-drying in 1.4ml of 0.1M-ammonium acetate buffer, pH 5.5, and applying it to a column (1.2cm×24cm) of Sephadex G-10 (Superfine), equilibrated in the same buffer. The peak of quinacillin-containing material was eluted together with a peptide peak, and considerably ahead of the position for free quinacillin.

Another portion of this material, containing 100nmol of quinacillin, was subjected to high-voltage electrophoresis at pH 6.5. A neutral band was observed; on prolonged (3h) electrophoresis partial separation into two bands was obtained.

Thus quinacillin is shown to form a covalent attachment to native penicillinase during the enzyme-catalysed hydrolysis of quinacillin, the reaction being approximately stoicheiometric. This suggests that there is a covalent intermediate formed during the catalytic process and that quinacillin is bound to a protein side chain. This is supported by the demonstration of one or possibly two chymotryptic peptides that retain bound quinacillin. One plausible mode of attachment is an amide link between the β-lactam carboxyl and a lysine residue (Scheme 1). Several penicillins have been shown to yield stable penicilloamides on prolonged incubation at near-neutral pH with polylysine (Schneider & de Weck, 1966*b*) and derivatives of proteins obtained under similar conditions (Batchellor *et al.*, 1965) are probably also penicilloamides. However, it is well established that penicillins react with alcohols to form esters with the β-lactam carboxyl group (Mozingo & Folkers, 1949). The formation of an ester between quinacillin and a serine or threonine residue of penicillinase is therefore an alternative possibility to an amide derivative.

The formation of a covalent complex between quinacillin and penicillinase might occur at the active site as a result of interactions with a reactive amino acid side chain during the normal catalytic cycle. Alternatively, complex-formation might depend on non-productive binding either at the active site or at some other site. Although we cannnot rule out that attachment occurs at a hyper-reactive group distant from the active site, the reversibility of attachment to the native enzyme (Table 1) argues against this. On present evidence it is reasonable to conclude that the covalent complex is formed at or near the active site.

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