Reversible deactivation of β -lactamase by quinacillin

Extent of the conformational change in the isolated transitory complex

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1. Conditions have been established where the deactivation of the β -lactamase from *Staphylococcus aureus* PC1 by the penicillin substrate, quinacillin, is close to complete but fully reversible. 2. The temperature-dependence of the rate of re-activation indicated a half-life of about 170 min for the deactivated state at 0 °C. 3. Measurement of the relative viscosity of mixtures of enzyme and quinacillin at 8.4 °C ruled out any significant difference in shape or solvation between the deactivated and the normal enzyme. 4. C.d. measurements of the deactivated protein, separated from excess quinacillin, showed that the quinacillin side-chain chromophore was bound in an asymmetric environment. The ellipticity associated with the bound quinacillin chromophore decreased with the same first-order rate constant as that for reappearance of enzyme activity. These findings support the accumulation of a deactivated state that contains bound quinacillin or a derivative. 5. Quinacillin caused a 3-fold increase in the rate of ^3H exchange-out (at a rate that was low compared with that for the substantially unfolded or expanded protein). However, there was rapid exchange-out of about 50 ^3H atoms on addition of 1 M-urea to the deactivated enzyme, whereas the same concentration had no effect on the exchange-out of ^3H from native enzyme. 6. The interpretation that quinacillin increases the susceptibility of the native state to unfolding in the presence of urea is supported by the demonstration that SO_4^{2-} ions decreased the rate and extent of deactivation but had no effect on the rate of re-activation, as predicted from the observation that SO_4^{2-} ions, in competition with urea, stabilize the native state relative to the partially unfolded state H [Mitchinson & Pain (1985) J. Mol. Biol. 184, 331–342].

INTRODUCTION

The slow decrease in the catalytic activity of β -lactamase brought about by certain substrates has been explained both in terms of the chemical reactivity of a derivative of the substrate and in effects on the protein conformation (see the review by Pain & Virden, 1979). A conformational component in the deactivation of staphylococcal β -lactamase by quinacillin and by cephaloridine is supported by studies with antibodies (Carrey *et al.*, 1984), and conformational factors may explain the low reactivity of one acyl-enzyme species in the recently demonstrated branched catalytic pathway of cephalosporin hydrolysis (Faraci & Pratt, 1985).

The maximum extent of quinacillin-induced deactivation observed by Virden *et al.* (1978) in phosphate buffer was less than 80%, although the concentration of quinacillin was more than 60-fold greater than the apparent K_m in the slow phase of the reaction. This was interpreted in terms of an equilibrium between active and inactive states of the enzyme. However, it was noted that conversion into a partly active state could not be excluded. The following results confirm the former interpretation: deactivation in other buffers, though fully reversible, approaches completion; the kinetics of deactivation and of re-activation fit, in detail, the expectations deriving from the assumption that there is a single deactivated species with less than 5% of the activity of the native enzyme. This, together with the low rate of re-activation near 0 °C, has allowed us to

characterize the deactivated state by means of several physical probes.

EXPERIMENTAL

Materials and buffers

The extracellular β -lactamase from *Staphylococcus aureus* PC1 was isolated and purified as described by Robson & Pain (1976a) to give a specific activity of 115–140 kat/mol. Quinacillin was a gift from the Boots Co., Nottingham, U.K. Benzylpenicillin (potassium salt) was from BDH Chemicals, Poole, Dorset, U.K. Buffers were adjusted at 20 °C to the stated pH with either HCl or NaOH as appropriate after addition of any other compounds.

Unless otherwise stated, enzyme was deactivated by incubating enzyme and quinacillin in 0.1 M-Pipes/NaOH buffer, pH 6.8, containing 90 mM-NaCl and 1 mM-EDTA for 5 min at 25 °C.

Measurement of enzyme activity

For pH-stat measurements, substrates were dissolved in a solution containing 0.1 M-NaCl and 1 mM-EDTA. The reaction was followed by measuring the addition of 10 mM-NaOH from a digital burette. In most cases the results were recorded at 1 s intervals by a data logger (Persaud & Virden, 1984), and progress curves were corrected by the subtraction of a control curve recorded before addition of enzyme.

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Fig. 1. Biphasic kinetics of quinacillin hydrolysis by β -lactamase

Solutions contained 90 mM-NaCl and 1 mM-EDTA. The reaction was started by the addition of 5 μ l of a solution of β -lactamase in 10 mM-Pipes buffer, pH 6.8 (6.3 mg/ml), to 3 ml of 1 mM-quinacillin in the pH-stat cell at 25.1 $^{\circ}$ C at pH 6.8. Experimental values ($\bullet$) were corrected by subtraction of a control curve (enzyme absent). The continuous line shows the best fit to eqn. (1).

Absorbance measurements were made with the use of a 10 mm-light-path cuvette in the thermostatically controlled cell holder of a Cary model 210 spectrophotometer. The hydrolysis of benzylpenicillin was monitored by measuring the decrease in A_{240} (Samuni, 1975). Unless otherwise stated re-activation kinetics were measured at 25 $^{\circ}$ C in a solution containing 1 mM-benzylpenicillin, lysozyme (0.2 mg/ml), 90 mM-NaCl, 1 mM-EDTA and 10 mM-Pipes/NaOH buffer, pH 6.8.

Non-linear regression for estimation of kinetic parameters was carried out by using the program EO4HFF (Nottingham Algorithms Group) with analytically derived first and second derivatives.

C.d.

C.d. spectra were measured with a Jobin Yvon Dichrographe IV fitted with a thermostatically controlled cell housing. Spectra were recorded by a programmable data logger (K. C. Persaud & R. Virden, unpublished work).

Hydrogen exchange

Radioactivity present in vials containing 10 ml of Beckman MP scintillation fluid was measured with a Packard Tri-Carb 4000 scintillation counter correcting for quenching by means of external standards.

β -Lactamase was labelled by treatment with ethyl [$1\text{-}^{14}\text{C}$]acetimidate essentially as described by Davies & Virden (1979). A 100 μ l sample containing 1.2 mg of protein, dialysed against 1 M-N-ethylmorpholine/acetic acid buffer, pH 8.25, was added to 8.7 μ mol of ethyl [$1\text{-}^{14}\text{C}$]acetimidate (2.2 TBq/mol) dissolved in 10 μ l of a mixture of equal volumes of the same buffer and 1 M-NaOH. The protein (3.4 TBq/mol) was stored after

dialysis in 10 mM-Pipes/NaOH buffer, pH 6.8, containing 90 mM-NaCl and 1 mM-EDTA. Exchange-in was carried out as described by Kiener & Waley (1977) for β -lactamase I except that the protein solution, pH 7.6, was incubated with $^3\text{H}_2\text{O}$ for 15 h at 25 $^{\circ}$ C and then applied to a 15 cm $\times$ 2 cm column of Sephadex G-25 equilibrated with 0.1 M-NaCl in 0.1 M-Pipes/NaOH buffer, pH 6.6. The fraction that contained protein (0.3–0.5 mg/ml) was mixed with [^{14}C]enzyme (3 μ g/ml) and used immediately to monitor the course of exchange-out at 4.5 $^{\circ}$ C. Sufficient 0.3 M-quinacillin and 9.2 M-urea, in the same buffer, were added to give the required concentrations.

The protein present in samples taken during exchange-out was separated from solvent $^3\text{H}_2\text{O}$ at 0 $^{\circ}$ C by adsorption on three discs (25 mm diameter) of Whatman P11 paper. After being washed to remove the labelled solvent, the discs were incubated for 1 h at 18–20 $^{\circ}$ C with 1 ml of 2.5 M-NaCl in 50 mM-sodium formate buffer, pH 3.0, before radioactivity counting (Schrier, 1977). Dual-channel measurements of ^3H and ^{14}C provided the basis for determination of the H atoms/molecule (Barksdale & Rosenberg, 1982).

RESULTS AND DISCUSSION

Kinetics of quinacillin hydrolysis and of re-activation

In line with preliminary results (Pain & Virden, 1979), Fig. 1 shows that the time course of hydrolysis of quinacillin, monitored at a concentration more than 3-fold greater than the K_m (Virden *et al.*, 1978), closely fitted an exponential decrease (with rate constant k') from an initial velocity (v_i) to a final constant velocity (v_f) (eqn. 1):

$$[P]_t = v_f \cdot t - (v_f - v_i) \cdot (1 - e^{-k' \cdot t}) / k' \quad (1)$$

On dilution of a mixture of enzyme and quinacillin preincubated at 25 $^{\circ}$ C for 5 min into an excess of benzylpenicillin the subsequent time course of benzyl-

Fig. 2. Re-activation of quinacillin-deactivated β -lactamase

Enzyme (86 μ g/ml) was incubated with 5 mM-quinacillin and 1 mM-EDTA in 0.1 M-Mes buffer, pH 6.7, at 24.1 $^{\circ}$ C for 5 min and then diluted 250-fold into 3 mM-benzylpenicillin in the pH-stat cell at 24.1 $^{\circ}$ C. The symbols are as for Fig. 1.

Table 1. Kinetics of quinacillin hydrolysis by β -lactamase and of re-activation after dilution into 3 mM-benzylpenicillin

Kinetic constants were calculated by fitting the results shown in Figs. 1 and 2 to eqn. (1) as described in the text and in the Experimental section.

	v_i (kat/mol)	v_f (kat/mol)	$10^3 \times k'$ (s ⁻¹)
Deactivation (quinacillin hydrolysis)	3.72	0.03	64
Re-activation (benzylpenicillin hydrolysis)	7.80	64.7	1.8

Table 2. Extent of deactivation of β -lactamase by quinacillin in different buffers

Enzyme was incubated with quinacillin at the concentrations shown in a 0.1 M buffer at the temperature shown. With the exception of phosphate buffer, the solutions also contained 90 mM-NaCl and 1 mM-EDTA. After 5 min the solution was diluted 300- or 600-fold into 1 mM-benzylpenicillin for measurement of the re-activation kinetics as described in the Experimental section.

Buffer	[Quinacillin] (mM)	[Enzyme] (mg/ml)	Temp. (°C)	Deactivation (%)
Phosphate	5	0.162	25	60
Pipes	5	0.075	25	96
	0.05	0.016	25	85
	0.05	0.016	0	97
Mes	200	10	0	89
	5	0.075	20	97
Hepes	5	0.065	25	>98
Bistris	5	0.078	25	>95
	50	20	8	96
Triethanol- amine	5	0.080	25	97
	20	3	25	94

penicillin hydrolysis also closely fitted eqn. (1), with $v_f > v_i$ reflecting an exponential increase from the initial velocity to the final steady-state velocity (Fig. 2). The estimates of v_i , v_f and k' obtained by fitting the results shown in Figs. 1 and 2 are given in Table 1. The rate of re-activation, monitored in the presence of benzylpenicillin, was $1.5 \times 10^{-3} \pm 0.1 \times 10^{-3} \text{ s}^{-1}$ (mean $\pm$ S.D. for five samples) over the concentration range 2.5–10 mM and the rate was not significantly dependent on concentration.

The value of v_f for benzylpenicillin hydrolysis determined after dilution of the deactivated enzyme into the pH-stat cell (Table 1) represents approx. 50% of the normal activity of β -lactamase. However, dilution of enzyme plus quinacillin into benzylpenicillin led to at least 95% re-activation when the reaction was monitored spectrophotometrically and protein (lysozyme) was present in addition to the catalytic concentration of β -lactamase. The extent of deactivation was greater than 95% when the molar ratio of quinacillin to enzyme was large, and this extent was not significantly dependent upon buffer type, except that, as previously reported (Virden *et al.*, 1978), a smaller percentage deactivation was observed with phosphate (Table 2).

Scheme 1.

The above results support a model in which, in the presence of quinacillin, there is almost complete conversion of one species of the active enzyme into an essentially inactive state and in which a single inactive species relaxes to active enzyme when excess quinacillin is eventually exhausted. In particular, the observed ratio v_i/v_f for quinacillin hydrolysis (124) represents 99% deactivation, consistent with the value of 97% calculated from the ratio of 36 for the rate of deactivation and the rate of re-activation measured in the presence of benzylpenicillin (Table 1). However, it is not possible from these results to identify unequivocally the active and inactive species involved.

In Scheme 1, EQ represents the form or forms that may accumulate during the catalytic cycle before the slow deactivation process becomes significant. In the case of a slowly hydrolysed substrate such as quinacillin this is likely to be an acyl-enzyme (Coulson, 1985). If first-order deactivation reflects conversion of EQ into EQ' the re-activation rate will correspond to the conversion of EQ' into EQ if EQ' is a dead-end complex. Alternatively, EQ' may decompose to E', in which case re-activation will mirror the decay of quantitatively the most significant deactivated species, either EQ' or E'. A further possible mode of deactivation could arise from a pre-existing equilibrium between E and E', and this would depend on the preferential binding of Q to E'.

The essentially complete deactivation achieved as described above points to the possibility of isolating the deactivated state for physical characterization. Persistence of the deactivated state for a significant time was demonstrated by measurement of the rate of re-activation as a function of temperature (Fig. 3). The activation energy of $102 \text{ kJ} \cdot \text{mol}^{-1}$ indicates a half-life of about 170 min at 0 °C. Similar results were obtained whether the re-activation was measured by means of the pH-stat or, at a lower benzylpenicillin concentration, by spectrophotometry.

Viscosity of deactivated enzyme

Conversion into the partly unfolded state H is accompanied by a 4-fold increase in the specific viscosity (Robson & Pain, 1976b). The reduced specific viscosity of enzyme in the presence of 50 mM-quinacillin in 0.1 M-Bistris/HCl buffer, pH 7.2, measured with a capillary viscometer (flow time for water 173 s) during the period of deactivation at 8.4 °C was 3.00, 3.13 and 3.00 ml/g at protein concentrations of 20, 8 and 5 mg/ml respectively. There were no significant changes during the period of observation 5–170 min (90 min at 8 mg/ml), although the percentage inactivation decreased from 96 at 12.5 min to 28 and 22 at 65 min and 115 min respectively. The reduced specific viscosity observed at 20 ml/mg corresponds to that expected for the native enzyme as against a value greater than 10 ml/g expected for state H.

Fig. 3. Arrhenius plot of the rate of re-activation of β -lactamase

Estimates of k' were obtained as described in the text. O, Enzyme was deactivated at the temperature shown and transferred into the pH-stat cell at the same temperature. Other conditions were as for Fig. 2. ●, Enzyme (190 μ g/ml) was deactivated at 0 °C and then diluted 300-fold for spectrophotometric analysis of re-activation at the indicated temperature (see the Experimental section).

These results rule out any substantial change in shape or solvation of the deactivated enzyme.

C.d. of deactivated enzyme

The c.d. spectrum of β -lactamase has a strong near-u.v. band resulting from tyrosine residues (tryptophan being absent), and the spectrum is very sensitive to conformational change in the enzyme (Carrey & Pain, 1978). Spectroscopic study of the deactivated enzyme was limited by the presence of the excess quinacillin that was needed to bring deactivation close to completion. The temperature-dependence of the rate of re-activation (Fig. 3) indicates that the deactivated state should persist after rapid separation at a temperature close to 0 °C. The following ion-exchange method was developed in order to achieve complete removal of free substrate and product. The cationic resin Dowex 1 was equilibrated with triethanolamine/HCl buffer so that quinacillin, or quinacilloic acid, would be bound in exchange for Cl⁻ ions while the positive charged β -lactamase would not interact with the resin. β -Lactamase (3 mg/ml) was inactivated with 20 mM-quinacillin, as described in the Experimental section, cooled in ice and then applied to a column (1 cm diam. $\times$ 1.2 cm) of Dowex 1 X2 (-400 mesh; Cl⁻ form), equilibrated at 4 °C with 0.1 M-triethanolamine/HCl buffer, pH 6.8. The column was eluted with a further 2 ml of the same buffer, and the fraction containing the protein (approx. 2.5 ml) was

collected within 4 min and kept in ice. The concentrations of protein and of the quinacillin side-chain chromophore were determined spectrophotometrically (Virden *et al.*, 1975). Control experiments showed that the amounts of resin used were capable of retaining, with no detectable leakage, quantities of quinacillin 10-fold greater than those present in the separation procedure. Typical experiments with either quinacillin-treated enzyme or untreated enzyme gave a 90% recovery of protein and a 10% decrease in enzyme activity (after re-activation where applicable). Table 3 shows that, for a number of separations, an average of 0.76 mol of quinacillin chromophore remained/mol of deactivated enzyme and that, on dilution into benzylpenicillin, the rate of re-activation was similar to that observed with deactivated enzyme that had not been subjected to separation.

The c.d. of the deactivated enzyme was measured at 2–4 °C within a few minutes after separation from excess quinacillin, the time being recorded with reference to the application of the deactivated enzyme to the column. In the 220 nm region the close similarity with the spectrum of untreated enzyme indicated that no gross change in protein conformation was associated with deactivation. A new peak of positive ellipticity, with a maximum at 334 nm, gradually decreased during incubation at 4 °C (Fig. 4). This feature could not be derived from a protein chromophore and may be assigned to the quinacillin side-chain group, which has a maximum absorbance at 326 nm (Richards *et al.*, 1963).

In a separate experiment c.d. was measured at 334 nm and, to allow for base-line instability, at 400 nm; readings were corrected for the contribution of the solvent in the same cuvette. At intervals between c.d. measurements samples were withdrawn for measurement of the enzyme activity with benzylpenicillin. The first-order kinetics of the decrease in ellipticity at 334 nm were closely paralleled by the decrease in the extent of deactivation (Fig. 5). The half-life of 28 min is considerably shorter than that predicted from Fig. 3. This may be explained simply if it is assumed that recovery of active enzyme from the major inactivated species EQ' occurs both through EQ and E' (Scheme 1). Non-productive binding of E' to benzylpenicillin would decrease the rate

Table 3. Extent of deactivation and kinetics of re-activation of β -lactamase separated from excess quinacillin

β -Lactamase (3 mg/ml) was deactivated with 20 mM-quinacillin as described in the Experimental section and separated from excess substrate and product as described in the text. A portion was diluted 50-fold in ice-cold 0.1 M-triethanolamine buffer, pH 6.8, and then diluted a further 600-fold for spectrophotometric determination of the re-activation kinetics in the presence of 1 mM-benzylpenicillin. For definitions of v_i , v_t and k' see eqn. (1). The ratio v_i/v_t for benzylpenicillin hydrolysis is a measure of the fractional degree of deactivation.

Separation	Molar ratio	Time (min)	v_i/v_t	$10^3 \times k'$ (s ⁻¹)
1	0.66	20	0.33	1.63
2	0.79	25	0.26	1.50
3	0.72	6	0.03	1.17
4	0.87	19	0.13	1.64

Fig. 4. C.d. spectra of deactivated β -lactamase as a function of time

A solution of enzyme, deactivated and separated from excess quinacillin as described in the text, was immediately transferred to a 1 cm-light-path cell for c.d. measurements. Age of isolated enzyme at start of scan: (a) 27 min; (b) 54 min; (c) 107 min; (d) 146 min; (e) 194 min.

Fig. 5. Comparison between ellipticity at 334 nm and extent of deactivation of β -lactamase

Enzyme was treated as for Fig. 4 before c.d. measurements at 2 °C (▲). The benzylpenicillinase activity (■) was determined by transferring 10 μ l samples into 3 ml of 1 mM-benzylpenicillin in 0.1 M-triethanolamine buffer, pH 6.8, at 1.5 °C for spectrophotometric determination of the initial velocity. The curves are normalized on the basis of semi-logarithmic plots, taking the reactions to be complete at 1300 min. The continuous line is the best fit calculated from the semi-logarithmic plot of the c.d. measurements.

of re-activation towards a limiting value equal to the rate of conversion of EQ' into EQ.

The possibility that the new peak represented substrate or product free in solution was ruled out by measurement of the c.d. of quinacillin and quinacilloic acid obtained by incubation with β -lactamase under conditions shown by Virden *et al.* (1978) to bring about complete hydrolysis (Fig. 6). The shape and peak positions of the spectra differed markedly in the range 300–400 nm, and the peak intensity of positive ellipticity was more than 30-fold greater in the deactivated enzyme than in either the substrate or the product. These observations indicate that the deactivated state of the enzyme accumulating under these conditions has the quinacillin side-chain chromophore bound to it in an environment substantially more asymmetric than that of either the substrate or the product in free solution. This provides strong evidence against the accumulation of E' as the major inactive species of Scheme 1. The region of the near-u.v. c.d. spectrum in which the protein side-chain chromophores absorb may also contain a contribution from the bound form of quinacillin. Because this contribution is unknown it is not possible to draw quantitative conclusions about changes in the environment of tyrosine residues. However, there is no evidence

Fig. 6. C.d. spectra of quinacillin and quinacilloic acid

C.d. was measured in a 1 mm-light-path cuvette thermostatically controlled at 25 °C. In each experiment the concentration of quinacillin or quinacilloic acid was determined from A_{326} and spectra were corrected for the contributions of solvent (0.1 M-phosphate buffer, pH 6.8) and, where applicable, of β -lactamase. Spectrum A, 1 mM-quinacillin; spectrum B, quinacilloic acid obtained from 1 mM-quinacillin by incubation with β -lactamase (73 μ g/ml) for 5 h at 25 °C.

for the complete loss of the ellipticity associated with tyrosine residues during the process of deactivation and re-activation (Fig. 4). By contrast, a relatively small conformational change resulting in the folding intermediate state I leads to complete loss of ellipticity in this region (Craig *et al.*, 1985).

Hydrogen exchange from deactivated enzyme

Hydrogen-exchange measurements have been shown to be capable of demonstrating small changes in protein conformation or conformational dynamics (Woodward & Hilton, 1979). The effect of quinacillin on the rate of exchange-out of ^3H from the staphylococcal enzyme was determined (Fig. 7). In controls the reasonable fit to first-order kinetics of the average rate of exchange-out of approximately 100 ^3H atoms [4.0×10^{-4} and $6.6 \times 10^{-4} \text{ min}^{-1}$ for Figs. 7(a) and 7(b) respectively] probably reflects the exchange of hydrogen atoms from state N, with the majority of hydrogen atoms involved exchanging at similar rates. The possibility that the observed loss of ^3H is limited by the rate of exchange between states N and H is unlikely, since the rate of unfolding of staphylococcal β -lactamase has been estimated to be 10^{-15} s^{-1} at 25 °C, and an even lower value would be expected at 4.5 °C (Mitchinson & Pain,

Fig. 7. Effects of quinacillin and urea on hydrogen exchange-out kinetics of β -lactamase

The extent of exchange-out was determined as described in the Experimental section. At the time shown by the arrow, additional components were added. (a) Addition: ●, none; ▲, 20 mM-quinacillin; ▼, 20 mM-quinacillin and 1 M-urea. (b) Addition: ●, none; ▲, 1 M-urea.

1985). Addition of quinacillin brought about a modest increase in the rate of exchange of at least 40 ^3H atoms to an average rate of $1.8 \times 10^{-3} \text{ min}^{-1}$. The further addition of urea to 1 M led to the rapid exchange-out of about 50 ^3H atoms. The rate of exchange-out of the remaining ^3H atoms ($3.5 \times 10^{-3} \text{ min}^{-1}$) was little greater than that brought about by the addition of quinacillin alone. The rate of exchange-out of ^3H atoms in the presence of 1 M-urea, but in the absence of quinacillin, was $8.7 \times 10^{-4} \text{ min}^{-1}$, not significantly different from controls. Addition of 3.5 M-urea, which leads to

Table 4. Effect of Na_2SO_4 and NaCl on the kinetics of quinacillin hydrolysis by β -lactamase

β -Lactamase (1.66 $\mu\text{g}/\text{ml}$) was incubated with 5 mM-quinacillin in the pH-stat cell as described in the Experimental section. The numbers of separate experiments are shown in parentheses and, where appropriate, means $\pm$ S.D. are given. For definitions of v_i , v_f and k' see eqn. (1). The ratio v_f/v_i for quinacillin hydrolysis is a measure of the fractional residual activity.

Addition	v_i (nmol/s)	$10^2 \times v_f$ (nmol/s)	v_f/v_i	$10^2 \times k'$ (s^{-1})
None (1)	4.7	2.6	0.007	8.8
1.5 M- NaCl (3)	1.2 ± 0.1	14.0 ± 0.9	0.115	2.10 ± 0.08
0.5 M- Na_2SO_4 (3)	1.8 ± 0.1	42.9 ± 1.9	0.240	1.41 ± 0.02

complete unfolding of the protein, induced much more rapid exchange-out (K. C. Persaud, unpublished work). It may therefore be concluded that the deactivated enzyme possesses a native-like conformation that is not in rapid equilibrium with any substantially unfolded state. However, the deactivated enzyme is considerably more sensitive than the native enzyme to the action of 1 M-urea.

These results support the interpretation by Carrey *et al.* (1984) of experiments with antibodies that modified the activity of the deactivated enzyme. The effect on the quinacillin-deactivated enzyme was different from that on the cephaloridine-deactivated enzyme (delaying and accelerating re-activation respectively). These findings could not be explained by a simple shift in the equilibrium between a normal catalytic intermediate and a low-activity form of the enzyme, and it was suggested that the antibodies were altering the conformation of the catalytic or binding sites or modifying the conformational mobility in response to substrate. The present findings indicate that only small changes in conformation or dynamics are involved.

Rather different conclusions have been reached for β -lactamase I from *Bacillus cereus* on the basis of the effect of guanidinium chloride on the activity of the deactivated enzyme (Kiener *et al.*, 1980) and on a 25-fold increase in the rate of exchange-out of approximately 35 ^3H atoms in response to addition of a deactivating substrate to the native enzyme (Kiener & Waley, 1977). It was suggested that the deactivated enzyme is an unfolded derivative of the catalytically productive acyl-enzyme.

Sulphate decreases deactivation

The interpretation that quinacillin renders the native-like state of the deactivated enzyme susceptible to unfolding in the presence of urea may be tested by making use of the results reported by Mitchinson & Pain (1985). SO_4^{2-} ions stabilize the native state (N) relative to state H by strengthening hydrophobic interactions in state N. This is reflected in a decrease in the rate of unfolding but little effect on the rate of refolding. As shown by measurement of the rate of hydrogen exchange, quinacillin destabilized the native state and increased its susceptibility to urea. If the transition to this

Fig. 8. Protection by sulphate against deactivation of β -lactamase by quinacillin

A 10 μl portion of enzyme solution (63 $\mu\text{g}/\text{ml}$), deactivated by 5 mM-quinacillin in the presence of the indicated concentration of Na_2SO_4 , was added to 3 ml of 1.4 mM-benzylpenicillin in the pH-stat cell. The initial velocity of benzylpenicillin hydrolysis is shown as a percentage of the velocity observed with untreated enzyme. Further details are in the Experimental section.

quinacillin-induced state, N^*Q , involves the breaking of hydrophobic interactions, then SO_4^{2-} ions should stabilize state N relative to state N^*Q and lead to a decreased rate and extent of inactivation but to little or no effect on the rate of re-activation. These expectations were fulfilled. The initial velocity of quinacillin hydrolysis was decreased in the presence of Na_2SO_4 , but the enzyme was markedly protected against deactivation. NaCl at an equivalent ionic strength brought about a similar effect on the initial velocity but a smaller effect on the extent of deactivation (Table 4). When quinacillin-treated enzyme was diluted 600-fold into 1 mM-benzylpenicillin the rate of re-activation was independent of the presence of 0.5 M- Na_2SO_4 in the benzylpenicillin solution. However, the initial velocity of benzylpenicillin hydrolysis by quinacillin-treated enzyme was markedly increased when Na_2SO_4 was present in the initial incubation with quinacillin (Fig. 8).

Conclusions

The kinetics of quinacillin hydrolysis are similar to those observed with A-type substrates of the *B. cereus* β -lactamase I, where chemical and immunological evidence pointed to effects on the protein conformation (Citri *et al.*, 1976). The extent of the conformational change has often been regarded as being considerable; for example, see the review by Fink (1985). The present evidence shows that quinacillin potentiates the action of urea on staphylococcal β -lactamase without significant

perturbation of the protein conformation. The view that reversible unfolding is the primary event in deactivation of *B. cereus* β -lactamase I may need to be reassessed.

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