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Nucleophilic re-activation of the PC1 β-lactamase of *Staphylococcus aureus* and of the DD-peptidase of *Streptomyces* R61 after their inactivation by cephalosporins and cephamycins

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It has been shown previously [Faraci & Pratt (1985) Biochemistry **24**, 903–910; (1986) Biochemistry **25**, 2934–2941; (1986) Biochem. J. **238**, 309–312] that certain β-lactam-processing enzymes form inert acyl-enzymes with cephems that possess good leaving groups at the C-3′ position. These inert species arise by elimination of the leaving group from the initially formed and more rapidly hydrolysing acyl-enzyme, which has the 'normal' cephalosporoate structure. The present paper shows that a strong nucleophile, thiophenoxide, can catalyse the re-activation of three examples of these inert acyl-enzymes, generated on reaction of cephalothin and cefoxitin with the PC1 β-lactamase of *Staphylococcus aureus* and of cephalothin with D-alanyl-D-alanine transpeptidase/carboxypeptidase of *Streptomyces* R61. In view of the reversibility of the elimination reaction, demonstrated in model systems [Pratt & Faraci (1986) J. Am. Chem. Soc. **108**, 5328–5333], this catalysis is proposed to arise through nucleophilic addition to the exo-methylene carbon atom of the inert acyl-enzyme to regenerate a more rapidly hydrolysing normal cephalosporoate. Strong support for this scenario was obtained through comparison of the kinetics of the catalysed re-activation reaction with those of turnover of the relevant 3′-thiophenoxycephems, thiophenoxycephalothin and thiophenoxycefoxitin. The enzymes appear to stabilize the products of the elimination reaction with respect to the normal cephalosporoate, but more strongly to destabilize the transition states. The effects of other nucleophiles, including cysteine, glycine amide and imidazole, on the above enzymes and on other β-lactamases can be understood in terms of the model reaction kinetics and thermodynamics.

INTRODUCTION

Cephalosporins (Ia) and cephamycins (Ib) with good 3′-leaving groups, X, are known to inhibit certain β-lactamases transiently by formation of hydrolytically inert acyl-enzymes, for which the structures (IIIa) and (IIIb) respectively have been proposed (Faraci & Pratt, 1985, 1986*a*). These species can be seen to arise by elimination of X from the directly formed and more rapidly hydrolysing acyl-enzymes, (II) (Scheme 1). Similarly, the D-alanyl-D-alanine transpeptidase/carboxypeptidase (abbreviated DD-peptidase below) of *Streptomyces* R61, a model for the D-alanyl-D-alanine transpeptidases of bacterial cell walls (Ghuysen *et al.*, 1979), forms inert acyl-enzymes with cephalosporins (and hence the antibiotic properties of these compounds) that are more resistant to hydrolysis after elimination of a 3′-leaving group (Faraci & Pratt, 1986*b*). This mechanism by which more stable acyl-enzymes can be generated may be common to all serine β-lactamases and DD-peptidases, and may thus represent another factor that should be considered in the design of cephem antibiotics.

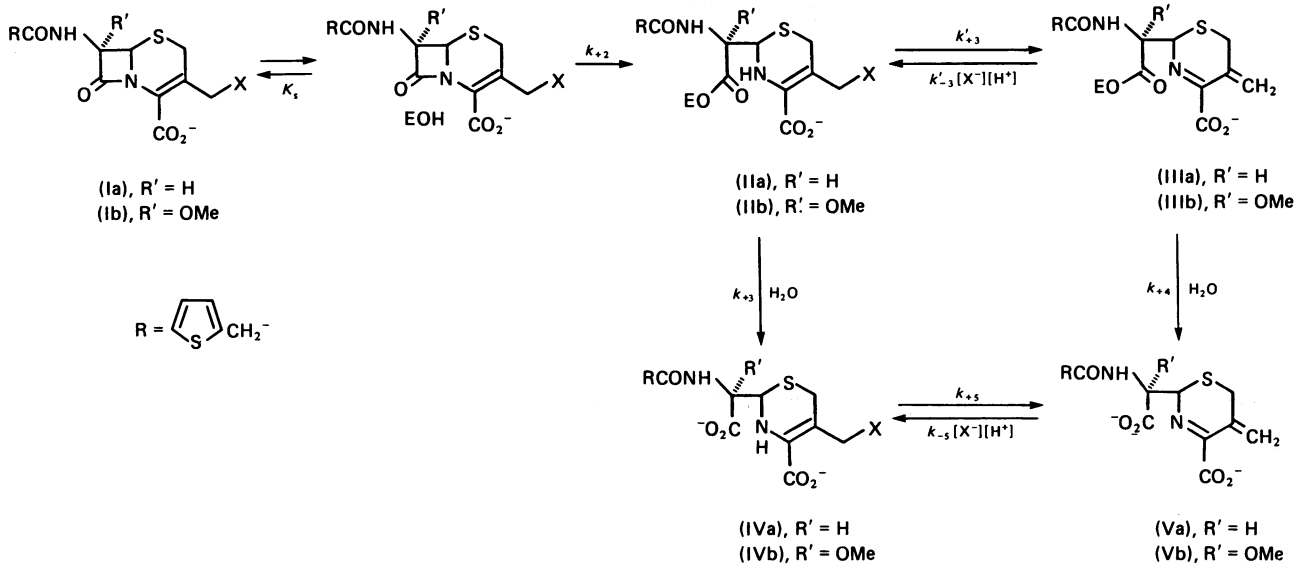
The structures of the hydrolysed products (IV) and (V) have been conclusively demonstrated (Faraci & Pratt, 1984; Pratt & Faraci, 1986), but those of the enzyme-bound species are necessarily somewhat more tenuous, although it is clear that (IIIa) and (IIIb) differ from (II) through the loss of the 3′-leaving group (Faraci & Pratt, 1985, 1986*a*).

We (Pratt & Faraci, 1986) and others (Buckwell *et al.*, 1986) have shown that elimination of the 3′-leaving group is reversible in free solution, i.e. (IV) and (V) of Scheme 1 are in equilibrium. If this is also true of the enzyme-bound species, i.e. if X^- can add to (III), regenerating (II), then Scheme 1 would predict that suitably nucleophilic X^- should be able to re-activate these enzymes through regeneration of the more labile (II). In the present paper we show that one strong nucleophile, thiophenoxide, can in fact re-activate (IIIa) (generated from the *Staphylococcus aureus* PC1 β-lactamase and from the *Streptomyces* R61 DD-peptidase) and (IIIb) (generated again from the PC1 β-lactamase) in a way consistent with Scheme 1. Our measurements also allow a view of how these enzymes affect the kinetics and thermodynamics of the elimination reaction.

EXPERIMENTAL

Materials

The PCI β-lactamase from *Staph. aureus*, the TEM-2 β-lactamase from *Escherichia coli* W 3310 and the β-lactamase of *Enterobacter cloacae* P99 were obtained from the Centre for Applied Microbiology and Research, Porton Down, Wilts. U.K., and used as supplied, as previously (Faraci & Pratt, 1985, 1986*a*). The soluble D-alanyl-D-alanine transpeptidase/carboxypeptidase of *Streptomyces* R61 was generously given by Dr. J.-M. Ghuysen and Dr. J.-M. Frère. Cephalothin was a gift from Eli Lilly and Co., and cefoxitin from Merck, Sharp



Scheme 1. Complete mechanism of turnover of cephems with 3′-leaving groups by β-lactamases and DD-peptidases

and Dohme. Nitrocefin was generously provided by Glaxo Research Ltd. Thiophenoxycephalothin [(Ia), X = SPh] was available from previous experiments (Pratt & Faraci, 1986). Thiophenol, which was purified by distillation under reduced pressure before use, was purchased from Aldrich Chemical Co.

Thiophenoxycefoxitin [(Ib), X = SPh] was prepared by the general procedure of Shimizu *et al.* (1976). Sodium cefoxitin (1.0 g, 2.2 mmol) was added to 12.0 ml of 1.0 M-potassium phosphate buffer, pH 7.10, containing 0.267 g of $NaHCO_3$, and held, with stirring, at 95 °C. Thiophenol (0.45 ml, 44 mmol) was then added immediately. After 75 min, the solution was cooled to room temperature and the excess thiophenol extracted with ethyl acetate. The aqueous layer was then acidified to pH 2 and the cephalosporin extracted with ethyl acetate. Purification was effected by way of the benzhydryl ester, which was prepared by the general procedure previously described (Faraci & Pratt, 1986*a*), and eluted with benzene/ethyl acetate (11:1, v/v) from a silica-gel column (2 cm × 22 cm). Further purification was achieved by preparative t.l.c. on silica gel, where the required ester had an R_F of 0.35 when the plate was developed with benzene/ethyl acetate (9:1, v/v). Cleavage of the benzhydryl ester with trifluoroacetic acid was achieved as also described previously (Faraci & Pratt, 1986*a*). Finally, the required product, as its sodium salt, in 5% overall yield, was eluted with water from a Sephadex G-25–40 column (1.0 cm × 22 cm), and isolated in solid form by freeze-drying. The ^1H n.m.r. spectrum of this material, which showed no sign of cefoxitin, was as follows: δ (p.p.m.) (^2H_2O) 3.18, 3.61 (2 H, ABq, J = 18 Hz, 2-*H*), 3.50 (3 H, s, OCH_3), 3.53, 4.37 (2 H, ABq, J = 14 Hz, $-CH_2S-$), 3.97 (2 H, s, Th-CH_2), 4.98 (1 H, s, 6-*H*), 7.08 (2 H, m, Th-3′-*H* + Th-5′-*H*) and 7.34–7.50 (6 H, m, Th-4′-*H* + −S*Ph*). Alkaline hydrolysis of this material yielded, according to absorption spectra, 1 equivalent of thiophenoxide; assay of the hydrolysis mixture with Ellman's reagent (see below) indicated the presence of 1 equivalent of thiol.

Analytical methods

All experiments involving the PC1 β-lactamase were performed at 20 °C in 0.1 M-potassium phosphate buffer, at pH 7.5, and those with the TEM-2 and *Ent. cloacae* P99 β-lactamases were carried out in a similar buffer at pH 7.0 at 30 °C, unless otherwise stated. Experiments with the R61 DD-peptidase were routinely carried out in 20 mM-potassium phosphate buffer, pH 7.0, at 37 °C. Absorption spectra and steady-state reaction rates were measured by means of a Cary 219 spectrophotometer. β-Lactamase activity was routinely assayed against benzylpenicillin by the spectrophotometric method of Waley (1974), and that of R61 DD-peptidase was determined against hippuryl-DL-phenyl-lactate (Govardhan & Pratt, 1987; Faraci & Pratt, 1986*b*). The enzyme concentrations were determined spectrophotometrically, with assumptions with respect to absorption coefficients as made previously (Faraci & Pratt, 1986*a*,*b*). The active-site concentration of the PC1 β-lactamase was also determined by spectrophotometric measurement (260 nm, $\Delta\epsilon = 7800\ M^{-1}\cdot cm^{-1}$) of the stoichiometric burst on its interaction with cephalothin (Faraci & Pratt, 1985). The absorption coefficient at 412 nm of the 2-nitro-5-mercaptobenzoate dianion was assumed to be $13600\ M^{-1}\cdot cm^{-1}$ (Ellman, 1959).

Re-activation of the PC1 β-lactamase and the R61 DD-peptidase by thiophenol

The inactive acyl-enzyme (IIIa) of the PC1 β-lactamase was generated by reaction of the enzyme (10.0 μM) with cephalothin (10.4 μM). A 15 μl portion of this solution was then immediately diluted into an assay mixture (0.5 ml) in a cuvette, containing the good substrate nitrocefin (3 mM) and various concentrations of thiophenol (0–10 mM). The initially measured activity was small but it increased with time, corresponding to hydrolysis of (IIIa), and was monitored by the absorption changes at 486 nm accompanying nitrocefin hydrolysis. First-order rate constants of re-activation [hydrolysis of

(IIIa)] at each thiophenol concentration could then be calculated from these data by a previously described method (Faraci & Pratt, 1985). Similarly, the acyl-enzyme (IIIb) was generated by reaction of the PC1 β-lactamase (9.4 μM) with cefoxitin (300 μM). Again the return of activity in the presence of thiophenol (0–1.4 mM) was monitored, in this case by measuring benzylpenicillin (2 mM) hydrolysis at 232 nm. The dilution in this case was 5 μl to 0.4 ml.

Control experiments were performed with both of these acyl-enzymes, where re-activation was followed in the presence of various concentrations of phenol (0–5.0 mM).

The inert acyl-enzyme (IIIa) of the R61 DD-peptidase was generated by reaction of the enzyme (7.0 μM) with cephalothin (10.0 μM). After complete inactivation was achieved, a small amount (final concn. 0.15 μM) of the TEM-2 β-lactamase was added in order to destroy excess cephalothin. Portions of this acyl-enzyme solution were then incubated with solutions containing various concentrations (0–2.0 mM) of thiophenol. Samples of the incubation mixture were taken at appropriate intervals and assayed for DD-peptidase activity against hippuryl-DL-phenyl-lactate. First-order rate constants of re-activation could be calculated directly from these data.

Determination of the rate constants for elimination of thiophenoxide from the acyl-enzymes (II)

The PC1 β-lactamase (17–22 μM) was incubated with cefoxitin (3 mM) until the steady state was reached (10 min). Thiophenol (final concn. 0.4–0.5 mM) in acetonitrile (final concn. 0.3%, v/v) was then added and the incubation continued for a further 10 min. At this time a small amount of Ellman's reagent (to a final concentration of 0.8 mM) was added and the increase in absorbance at 412 nm, corresponding to release of thiophenol from (IIb), was monitored. First-order rate constants, which were independent of the concentration of Ellman's reagent, could be calculated from these data. A similar experiment was performed with the R61 DD-peptidase, where the concentration of the enzyme, cephalothin, thiophenol and Ellman's reagent were 13.5 μM, 20 μM, 150 μM and 0.7 mM respectively.

A similar procedure was used to determine the rate constants for elimination of thiophenol from the adducts (IV) in free solution. Equilibrium mixtures were diluted into solutions of Ellman's reagent, yielding instantaneous absorption at 412 nm, corresponding to the free thiophenol, followed by an exponential appearance of absorption, corresponding to dissociation of (IV). From these data both equilibrium and rate constants could be calculated.

Pre-steady-state kinetics

The kinetics of formation of (IIIb) from thiophenoxycefoxitin and the PC1 β-lactamase could be determined from the decrease in immediately active enzyme as a function of time. Thus portions of a reaction mixture containing the enzyme (7.4 μM) and various concentrations of thiophenoxycefoxitin (0–2.0 mM), 1.0 min after mixing, were added to a standard benzylpenicillin assay mixture. The initial velocity of benzylpenicillin hydrolysis in each case was taken as a measure of the amount of immediately active enzyme remaining, i.e. of all forms except (IIIb).

The amplitudes and first-order rate constants of the burst of thiol release on formation of (IIIa) and (IIIb) from thiophenoxycephalothin and thiophenoxycefoxitin respectively could be determined from measurements in the presence of Ellman's reagent. In one series of experiments 10 μl portions of PC1 β-lactamase solution (final concn. 0.070 μM) were added to 0.4 ml samples of thiophenoxycephalothin (final concns. 0.3–1.25 mM) containing Ellman's reagent at a final concentration of 0.4 mM. The ensuing thiophenol release was monitored at 412 nm (2-nitro-4-mercaptobenzoate). Similar experiments were performed with the PC1 β-lactamase and thiophenoxycefoxitin where the concentration of the latter in separate runs was 0.28 mM and 0.53 mM.

Effects of other nucleophiles

The acyl-enzymes formed on the interaction of the PC1 β-lactamase and thiophenoxycephalothin and thiophenoxycefoxitin were also exposed to other nucleophiles, and, as described above, the return of activity corresponding to hydrolysis of the acyl-enzyme was monitored. The nucleophiles tested in this way were 2-mercaptoethanol, glycine amide, 3,4-lutidine and imidazole, each at 0.1 M concentration in the reaction mixture. Also by following the procedures described above, the re-activation of the cephalothin-inactivated R61 DD-peptidase was studied in the presence of 10 mM each of 2-mercaptoethanol, 3,4-lutidine, cysteine and imidazole.

Effects of thiophenol on the turnover rates of cephalothin and cefoxitin by other β-lactamases

These were determined by steady-state experiments with the TEM-2 and *Ent. cloacae* P99 β-lactamases. Thus 5 μl of an enzyme solution (yielding final concentrations of 0.60 μM and 0.73 μM for the TEM-2 and P99 β-lactamases respectively) was added to 0.40 ml of cefoxitin solution (180 μM) containing 0.5 mM-thiophenol (and 0.25% acetonitrile, the vehicle of thiophenol addition). The ensuing cefoxitin hydrolysis was monitored in a 2 mm-pathlength cuvette at 260 nm. Similar experiments were performed with 310 μM-cephalothin, where the TEM-2 and P99 β-lactamase concentrations were 7.1 nM and 5.8 nM respectively.

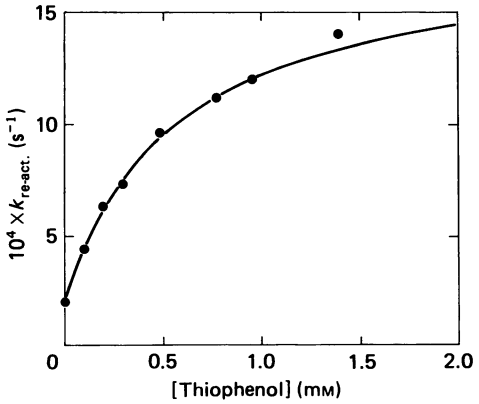


Fig. 1. Rate constants of re-activation in the presence of thiophenol of the inert acyl-enzyme formed on interaction of cefoxitin and *Staph. aureus* PC1 β-lactamase

Reaction conditions are given in the Experimental section.

RESULTS

Staphylococcus aureus PC1 β-lactamase, cefoxitin and thiophenol

In the presence of thiophenol, the inert acyl-enzyme (IIIb), formed on reaction of the PC1 β-lactamase with cefoxitin, regenerated free enzyme, i.e. re-activated, more rapidly than in its absence. A non-linear relationship was observed (Fig. 1) between the rate of re-activation and the total thiophenol concentration, tending towards saturation at the higher thiophenol concentrations. Phenol, at concentrations up to 5 mM, at which it does not add to (V) [as shown by ^{1}H-n.m.r. observations similar to those described elsewhere (Pratt & Faraci, 1986)], had no effect on the rate of re-activation of (IIIb). The thiophenol effect, therefore, probably does not result from non-specific binding to the enzyme.

These results were interpreted in terms of Scheme 1, where the pseudo-first-order rate constant for re-activation of (IIIb), $k_{re\text{-}act.}$, is given by eqn. (1):

$$k_{re\text{-}act.} = k_{+4} + \frac{k_{+3}[PhS^-]}{K'_3 + [PhS^-]} \quad (1)$$

if $k'_3 \gg k_{+3}$, i.e. the thiophenol addition is a pre-equilibrium $\{K'_3$ [the dissociation constant of (IIb)] $= k'_{+3}/k'_{-3}\}$ to hydrolysis (if this were not so, saturation in thiophenol would not be predicted by Scheme 1). Rearrangement of eqn. (1) yielded the reciprocal eqn. (2):

$$1/(k_{re\text{-}act.} - k_{+4}) = 1/k_{+3} + K'_3/k_{+3}[PhS^-] \quad (2)$$

The linear reciprocal plot (not shown), according to this equation, of the data of Fig. 1 gave the values for K'_3 and k_{+3} presented in Table 1. The value of k_{+4} required for this plot was taken from the $k_{cat.}$ values for cefoxitin and/or thiophenoxycefoxitin (see below). The continuous curve in Fig. 1 is calculated from eqn. (1) and these parameters.

The rate constant k'_{+3} could be determined directly by measurement of the rate of irreversible thiophenol release from (IIIb) (generated, as described in the Experimental section, from a mixture of the PC1 β-lactamase, cefoxitin and thiophenol) in the presence of Ellman's reagent as a thiol trap. Dilution of the reaction mixture into an Ellman's reagent solution led to instantaneous absorption at 412 nm (2-nitro-5-mercaptobenzoate), due to free thiophenol, followed by a first-order increase corresponding to elimination of thiophenol from (IIb). From this rate constant k'_{+3}, and the equilibrium constant K'_3 determined above, the rate constant for thiophenol addition to (IIIb), k'_{-3}, could be calculated; these constants are also shown in Table 1. The amplitude of the first-order reaction corresponded to 0.55 $[E]_0$ (where $[E]_0$ is the total active enzyme concentration), which is appropriate for the thiophenol concentration of 0.5 mM at which the experiment was conducted.

In order to confirm that Scheme 1 was indeed an appropriate framework in which to interpret these results, i.e. that thiophenol, and, by implication, other nucleophiles, could add to (III), complementary experiments were undertaken with thiophenoxycefoxitin [(Ib), X = SPh]. In these experiments (II) was derived from (I) rather than, as in the experiments described above, from (III). This led to, as described below, an independent

Table 1. Kinetic parameters relating to Scheme 1

The *Staph. aureus* PC1 β-lactamase experiments were carried out at pH 7.0 at 30 °C, and those of the *Streptomyces* R61 DD-peptidase at pH 7.0 at 37 °C. Abbreviation: N.D., not determined

		Parameters for substrate-turnover experiments			Parameters from thiophenol-elimination experiments				
Enzyme	Substrate	K_s (mM)	k_{+2} (s^{-1})	k_{+4} (s^{-1})	k_{+3} (s^{-1})	k'_{+3} (s^{-1})	k'_{-3} ($s^{-1}\cdot M^{-1}$)	K'_3 (mM)	k_{+5} (s^{-1})	k_{-5} ($s^{-1}\cdot M^{-1}$)	K_5 (mM)
PC1	Thiophenoxy-cephalothin	N.D. (< 0.1)	1.2–2.4	$(1.1\pm0.1)\times10^{-3}$	N.D. ($\geqslant k_{+2}$, $= 300k'_{+3}$)	N.D.	2.3±0.5	N.D.	0.07	500	0.14
PC1	Thiophenoxy-cefoxitin	2.0±0.4	0.067±0.007	$(2.8\pm0.5)\times10^{-4}$	$(1.5\pm0.5)\times10^{-3}$	$(6\pm1)\times10^{-3}$ $(4.4\pm1.0)\times10^{-3}$*	12±1	0.50±0.04	0.17	1100	0.15
R61	Thiophenoxy-cephalothin	N.D.†	N.D.†	$(3.1\pm0.3)\times10^{-6}$	$(3.6\pm0.4)\times10^{-5}$	$(6.6\pm0.8)\times10^{-4}$ $(5.8\pm0.2)\times10^{-4}$*‡	2.2±0.5	0.30±0.04	0.24	1100	0.25

* Determined from substrate-turnover experiments.
† $k_{+2}/K_s = 500\ s^{-1}\cdot M^{-1}$ (Faraci & Pratt, 1986*b*).
‡ Faraci & Pratt (1986*b*).

determination of k'_{+3}, which could then support or refute Scheme 1.

The steady-state kinetic parameters for cefoxitin and thiophenoxycefoxitin hydrolysis, catalysed by the PCI β-lactamase, were $K_m = 19 \pm 2\ \mu M$ and $k_{cat.} = (3.0 \pm 0.3) \times 10^{-4}\ s^{-1}$ and $K_m = 50 \pm 20\ \mu M$ and $k_{cat.} = (2.8 \pm 0.6) \times 10^{-4}\ s^{-1}$ respectively. Note that the kinetics for thiophenoxycefoxitin were determined under irreversible (with respect to thiophenol) conditions, i.e. in the presence of the thiol trap, Ellman's reagent (0.3 mM). Our prior work (Faraci & Pratt, 1985, 1986*a*) shows that Scheme 1 is appropriate for cefoxitin turnover by the PCI β-lactamase, with $k'_{+3} \gg k_{+3}$, the elimination of X^- ($NH_2CO_2^-$) irreversible, and k_{+4} ($= k_{cat.}$) rate-determining at saturation. The $k_{cat.}$ value for thiophenoxycefoxitin suggests that the hydrolysis of (IIIb), which should then be an intermediate common to thiophenoxycefoxitin and cefoxitin, is also rate-determining to its hydrolysis under conditions of enzyme saturation. This conclusion was confirmed by dilution of a PC1 β-lactamase/thiophenoxycefoxitin mixture into a benzylpenicillin assay mixture, whereupon an initial enzyme activity of zero was recorded, which increased with time with a rate constant of $(2.0 \pm 0.3) \times 10^{-4}\ s^{-1}$; identical results were obtained with cefoxitin. These steady-state results support the propositions that for thiophenoxycefoxitin $k'_{+3} \gg k_{+3}$, a result deduced from the thiophenol activation experiments described above (if this were not so then the $k_{cat.}$ values for thiophenoxycefoxitin should be larger than that of cefoxitin), and that $k'_{+3} \gg k_{+4}$.

On incubation of the PC1 β-lactamase and thiophenoxycefoxitin together, the amount of residual β-lactamase activity, A, determined by assay at any time t_c after mixing, should be given, according to Scheme 1, by eqn. (3):

$$A = A_0 \cdot \exp\{-k_2[S]_0 t_c/(K_s + [S]_0)\} \quad (3)$$

where A_0 is the intial enzyme activity, and $[S]_0$ (the initial substrate concentration) $\gg [E]_0$. It is assumed in the derivation of this equation that non-covalent binding ($1/K_s$) is fast and reversible, and that $k_2 \gg k_{+3}, k_{+4}$. The former assumption seems reasonable on the basis of all prior experiments (Anderson & Pratt, 1981, 1983; Faraci & Pratt, 1985, 1986a), but the latter, with respect to k_{+3} at least, is questionable, although self-consistent with all that follows. A linear plot of $t_c/\ln(A/A_0)$ versus $1/[S]_0$ (not shown) yielded the values of k_{+2} and K_s given in Table 1.

Also readily measurable in the pre-steady-state situation is the burst of thiophenol produced on mixing the PC1 β-lactamase and thiophenoxycefoxitin under irreversible (Ellman's reagent) conditions. Under these conditions (see the Experimental section for details) the thiophenol burst rate constant, k_{burst}, and amplitude, π, are given, according to Scheme 1, by eqns. (4) and (5) respectively:

$$k_{burst} = k_{+4} + \frac{k_{+2}k'_{+3}[S]_0}{(k_{+3} + k'_{+3})(K_s + [S]_0) + k_{+2}[S]_0} \quad (4)$$

$$\pi/[E]_0 = \frac{(k_{+2}[S]_0)^2(k_{+3} + k'_{+3})k'_{+3}}{\{k_{+4}(K_s + [S]_0)(k_{+3} + k'_{+3}) + k_{+2}[S]_0(k'_{+3} + k_{+4})\}^2} \quad (5)$$

Given values for k_{+2} and k_{+4} and the knowledge that $k'_{+3} \gg k_{+3}$, the value of k'_{+3} can be calculated from eqn. (4). This calculation yielded k'_{+3} values of $0.0045\ s^{-1}$ and $0.0043\ s^{-1}$ at the two substrate concentrations used, the average of which is reported in Table 1. These results show that at the substrate concentrations chosen $k_{+2}[S]_0 \gg k_{+4}(K_s + [S]_0)$ and hence, again given that $k'_{+3} \gg k_{+3}, k_{+4}$, the burst amplitude, π, according to eqn. (5) should be equal to the initial enzyme concentration $[E]_0$. The measured thiophenol (in the presence of Ellman's reagent) burst amplitudes were $(0.7 \pm 0.1)\ [E]_0$, in reasonable agreement with expectation and certainly not suggestive of a number greater than 1.

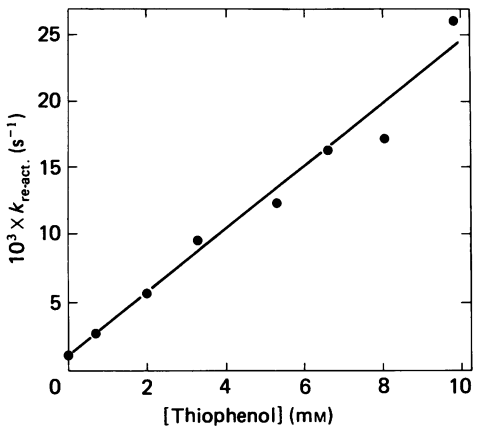


Fig. 2. Rate constants of re-activation in the presence of thiophenol of the inert acyl-enzyme formed on interaction of cephalothin and *Staph. aureus* PC1 β-lactamase

Reaction conditions are given in the Experimental section.

Staphylococcus aureus PC1 β-lactamase, cephalothin and thiophenol

Thiophenol also catalysed re-activation of the acyl-enzyme (IIIa) formed on interaction of the PC1 β-lactamase with cephalothin (Faraci & Pratt, 1985). Again phenol (to 5 mM) had no effect on the re-activation rates. In this case, however, the relationship between the pseudo-first-order reactivation rate constant and thiophenol concentration was linear to achievable concentrations (Fig. 2), and yielded an apparent second-order rate constant $k_{app.} = 2.3 \pm 0.5\ s^{-1} \cdot M^{-1}$. This situation of linear response could arise either because $K'_{+3} \gg [PhS^-]$ [see eqn. (1)], in which case $k_{app.} = k_{+3}/K'_{+3}$, or because $k_{+3} \gg k'_{+3}$, contrary to the assumption made in the derivation of eqn. (1), and in which case $k_{app.} = k'_{+3}$.

Distinction between these possibilities was achieved through further kinetics experiments, in this case with thiophenoxycephalothin. Thiophenol burst rates and amplitudes were again measured in the presence of Ellman's reagent. Since both were independent of $[S]_0$ in the concentration range studied, eqns. (4) and (5) could be reduced to eqns. (6) and (7):

$$k_{burst} = k_{+2}k'_{+3}/(k_{+2} + k_{+3} + k'_{+3}) \quad (6)$$

$$\pi/[E]_0 = 1 + k_{+3}/k'_{+3} \quad (7)$$

{The value of k_{+4} should be that ($0.0011\ s^{-1}$) determined from steady-state and return-of-activity experiments with cephalothin (Faraci & Pratt, 1985), and the required conclusion that $[S]_0 \gg K_s$ is not unreasonable in view of the K_s values previously obtained with similar cephalosporins (Faraci & Pratt, 1985)}. The thiophenol

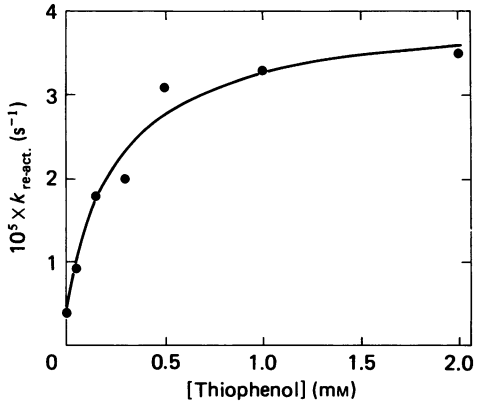


Fig. 3. Rate constants of re-activation in the presence of thiophenol of the inert acyl-enzyme formed on interaction of cephalothin and *Streptomyces* R61 DD-peptidase

Reaction conditions are given in the Experimental section.

(Ellman's reagent) burst amplitude of (300±20) $[E]_0$ thus gives the value of $k_{+3}/k'_{+3} = 300$. This result proves that $k_{app.} = k'_{-3}$ (Table 1).

The steady-state rate subsequent to the burst, which also did not vary with $[S]_0$ in these experiments, indicating that substrate saturation had been achieved, yielded a $k_{cat.}$ value of 0.17 s^{-1}. Since $k_{cat.} = (1 + k_{+3}/k'_{+3})k_{+4}$ (Faraci & Pratt, 1985), a k_{+4} value of 0.0011 s^{-1} (Table 1) could be calculated, which is in complete agreement with that derived previously from the cephalothin experiments referred to above.

A pre-steady-state measurement was also made on this system at 260 nm, where opening of the β-lactam ring is monitored. The concentrations of thiophenoxycephalothin and enzyme (100 μM and 6 μM respectively) are such that essentially all turnover should occur by hydrolysis of (IIa). Although the observations are complicated by the dissociation of (IVa) to (Va), which occurs at similar rates (Faraci & Pratt, 1986*b*), no clear burst of loss of the cephem chromophore was observed, suggesting that $k_{+2} \leqslant k_{+3}$. Application of this result to eqn. (6) leads to an estimate of k_2 of between 1.2 s^{-1} (assuming $k_{+2} \ll k_{+3}$) and 2.4 s^{-1} (assuming $k_{+2} = k_{+3}$). A value of k_{+2} of this order is in accord with previous observation (Anderson & Pratt, 1981; Faraci & Pratt, 1985), which also suggests that $k_{+2} \approx k_{+3}$ is reasonable.

Streptomyces R61 DD-peptidase, cephalothin and thiophenol

The inert acyl-enzyme of structure (IIIa), generated on interaction of the *Streptomyces* DD-peptidase and cephalothin, was also more rapidly restored to active enzyme in the presence of thiophenol, but not phenol. Saturation kinetics were observed in this case (Fig. 3), allowing the analysis applied to the PC1 β-lactamase/cefoxitin system to be used. This, along with direct measurements of the rate of thiophenol elimination from (IIa), yielded the values of k_{+3}, K'_3, k'_{+3} and k'_{-3} shown in Table 1. The value of k_{+4} also quoted in this Table is taken from the rate of re-activation of the enzyme–cephalothin complex, which is identical with the rate of reaction of the enzyme–thiophenoxycephalothin complex in the presence of Ellman's reagent (Faraci & Pratt, 1986*b*).

Other nucleophiles and other β-lactamases

The rate of regeneration of free enzyme from the PC1 β-lactamase–cephalothin complex (IIIa) was not accelerated by the presence of 2-mercaptoethanol, 3,4-lutidine, L-cysteine, glycine amide or imidazole at concentrations up to 0.1 M. On the other hand, the rate of re-activation of the R61 DD-peptidase–cephalothin complex was increased 3-fold by 10 mM-2-mercaptoethanol and some 50% by 10 mM-L-cysteine and -3,4-lutidine; 10 mM-imidazole, however, had little effect.

The effect of thiophenol on the steady-state rate of hydrolysis of cephalothin and cefoxitin by the TEM-2 and *Ent. cloacae* P99 β-lactamases was determined under the conditions described in the Experimental section. Acceleration (some 4-fold at 0.5 mM-thiophenol) was observed only in the case of TEM-2 β-lactamase and cefoxitin.

DISCUSSION

The experiments described above demonstrate that a strong nucleophile, thiophenoxide, is able to catalyse the hydrolysis of the inert acyl-enzymes of putative structures (IIIa) and (IIIb) derived from the *Staph. aureus* PC1 β-lactamase and the *Streptomyces* R61 DD-peptidase and thus regenerate the free enzymes. On the basis of analogy with the reaction of thiophenoxide with (V) in free solution (Pratt & Faraci, 1986; Buckwell *et al.*, 1986), the mechanism of this catalysis is likely to be that implied by Scheme 1, namely nucleophilic addition to the 3-exomethylene carbon atom of (III), followed by hydrolysis of the more labile acyl-enzyme (II); the greater rates of hydrolysis of species (II) from these enzymes have been previously demonstrated (Faraci & Pratt, 1985, 1986*a*,*b*).

That the mechanism of re-activation is indeed as shown in Scheme 1 is demonstrated by the close correlation between the results of experiments designed to generate the intermediate (II) in two ways, firstly by addition of thiophenol to species (III), formed by interaction of the enzymes with cephems with very good leaving groups, and secondly from the cephems thiophenoxycephalothin (Ia) (X = SPh) and thiophenoxycefoxitin (Ib) (X = SPh), which have the 3′-thiophenoxy substituent already in place. For example, the reactions of thiophenoxycefoxitin with the PC1 β-lactamase and of thiophenoxycephalothin with the R61 DD-peptidase appear to yield (III) stoichiometrically in a single turnover, i.e. $k'_{+3} \gg k_{+3}$, whereas (IIIa) is generated from the PC1 β-lactamase and thiophenoxycephalothin only to a small extent in steady state, i.e. $k_{+3} \gg k'_{+3}$. This difference between the response of the PC1 β-lactamase to the cephalosporin in one hand and the cephamycin on the other is not unexpected, since it is known that addition of a 7α-methoxy group to cephalosporins slows down deacylation of species (II) (Faraci & Pratt, 1986*a*). The significant point with respect to the current experiments, however, is that the same difference emerges from analysis of the thiophenol re-activation experiments, namely that in the cases of the PC1 β-lactamase and cefoxitin and of the R61 DD-peptidase and cephalothin saturation kinetics are obtained (Figs. 1 and 3), which are best interpreted in terms of rapid pre-equilibrium addition of thiophenol before the re-activation step, i.e., in terms of Scheme 1, $k'_{+3} > k_{+3}$, whereas with the PC1 β-lactamase and cephalothin no

Table 2. Effects of enzymes on the free-energy changes associated with the elimination reaction

The *Staph. aureus* PC1 β-lactamase experiments were carried out at pH 7.0 at 30 °C, and those of the *Streptomyces* R61 DD-peptidase at pH 7.0 at 37 °C. $\delta\Delta G_f^{\ddagger}$ and $\delta\Delta G_r^{\ddagger}$ refer to the forward reaction (elimination) and the reverse reaction (addition) respectively.

Enzyme	Substrate	$\delta\Delta G^0$ (kJ/mol)	$\delta\Delta G_f^{\ddagger}$ (kJ/mol)	$\delta\Delta G_r^{\ddagger}$ (kJ/mol)
PC1	Thiophenoxycephalothin	−5.5	+7.6	+13.0
PC1	Thiophenoxycefoxitin	−2.9	+8.0	+10.9
R61	Thiophenoxycephalothin	−0.4	+16.8	+17.2

saturation in thiophenol is observed (Fig. 2), which can also be interpreted in terms of the expected result in this case, that $k_{+3} > k'_{+3}$.

A more quantitative analysis of this point was made in the case of the PC1 β-lactamase/thiophenoxycefoxitin system. Analysis of the kinetics of re-activation of (IIIb) by thiophenol on one hand, and the pre-steady-state kinetics of the β-lactamase-catalysed hydrolysis of thiophenoxycefoxitin on the other, yielded very similar values (Table 1) for the one common rate constant that could be extracted from each, k'_{+3}, to prove that the identical species (IIb) was generated by both routes.

Another observation in support of the nucleophilic addition mechanism was that 1.5 mM-thiophenol did not cause any acceleration of the return of activity of the inert acyl-enzyme, presumably of structure (IIa) (X = H), formed on interaction of the R61 DD-peptidase and desacetoxycephalothin [(Ia), X = H]. Other possible mechanisms for the re-activation seem unlikely.

Given the validity of the interpretation of these results in terms of Scheme 1, a comparison can be made between the kinetics and thermodynamics of the elimination reaction at the active site of these enzymes with those in free solution. The data of Table 1 indicate that two enzymes, the PC1 β-lactamase and the R61 DD-peptidase, do perturb the thermodynamics and, more particularly, the kinetics of the elimination reaction. This point is shown more clearly in Table 2, where the effects of the enzyme on the free energies of the transition states ($\delta\Delta G^{\ddagger}$) and products ($\delta\Delta G^0$) of the elimination reaction at a constant thiophenol concentration of 0.5 mM are presented. In order to calculate these values, the free energies of the free (IV) and enzyme-bound adducts (II) were arbitrarily matched. Values of k'_{+3} and K'_3 for the PC1 β-lactamase–thiophenoxycephalothin reaction of $3.3 \times 10^{-3} s^{-1}$ and 1.4 mM respectively were calculated, assuming a reasonable (Faraci & Pratt, 1985) value of $1.0\ s^{-1}$ for k_{+3}. Table 2 shows that, although the elimination products have been slightly stabilized with respect to the adducts by the enzyme, the rate constants in both directions are significantly decreased with respect to the model reaction, and particularly in the case of the DD-peptidase. This indicates that some different interactions with the enzyme occur in the transition state compared with those in reactants or products. A variety of steric and/or stereoelectronic (Boyd & Lunn, 1979) factors could explain these differences.

With respect to other β-lactamases, it is immediately clear that the activating effects of thiophenol are not universal. Only in the case of the TEM-2 β-lactamase and cefoxitin is there evidence of enhanced turnover in the presence of thiophenol. This observation correlates of course with the demonstrated accumulation of (IIIb) in this system (Fisher *et al.*, 1980; Faraci & Pratt, 1986*a*). The absence of any effect with cephalothin most probably reflects the fact that k_{+4} or k_{+3} (X = OAc) $\gg k'_{-3}[PhS^-]$, although, even if this were otherwise, the effect of thiophenol may still not be seen since acylation may well be rate-determining and k_{+3} much greater than k'_{+3}. These factors may hold for the P99 enzyme also, although the existence of the effect of the 3′-elimination reaction has not yet been demonstrated with class C β-lactamases.

The ineffectiveness of other nucelophiles can be explained in large part by the results of model studies with (V) (Pratt & Faraci, 1986). At pH 7.5, at concentrations up to 0.1 M, imidazole and glycine amide did not appear to add to (V), at least in the time period necessary to enhance re-activation of the acyl-enzymes (III). 2-Mercaptoethanol did add to (V), but slowly, apparently too slowly to affect the PC1 β-lactamase species (III), but rapidly enough to accelerate the hydrolysis of the R61 DD-peptidase analogue. The previously published data (Pratt & Faraci, 1986) concerning 3,4-lutidine addition suggest that, if its rates of addition were decreased by the enzymes to the extent observed with thiophenol, then it too would probably not effectively catalyse re-activation of the PC1 β-lactamase. Thus, of the nucleophiles employed in this study, only thiophenol is a strong enough nucleophile at neutral pH, kinetically and/or thermodynamically, to add to the 3-methylene group of (III) sufficiently to accelerate re-activation. These considerations may in part explain why the absorption spectra of (IIIa), and (IIIb), in two specific cases at least [PC1 β-lactamase/cephalothin (Faraci & Pratt, 1985) and TEM-2 β-lactamase/cefoxitin (Faraci & Pratt, 1986*a*)], indicate the structure (III) rather than adducts with enzymic nucleophiles.

This work provides further support for Scheme 1 as the complete mechanism of turnover of cephems with 3′-leaving groups by β-lactamases and DD-peptidases. The continually compounding resemblances between β-lactamases and DD-peptidases (Tipper & Strominger, 1965; Pratt & Govardhan, 1984; Kelly *et al.*, 1986) should not go unremarked. Whether any β-lactamase or β-lactam-killing site is usefully re-activated *in vivo* after exposure to cephems by (intra- or inter-molecular) nucleophilic addition is a matter only for speculation at present.

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