Effect of the 3'-leaving group on turnover of cephem antibiotics by a class C β -lactamase

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It has been previously demonstrated for class A β -lactamases and the DD-peptidase of *Streptomyces* R61 that the presence of a leaving group at the 3'-position of a cephalosporin can lead to the generation of more-inert acyl-enzyme intermediates than from cephalosporins lacking such a leaving group, and thus to β -lactamase inhibitors and potentially better antibiotics. In the present work we extend this result to a class C β -lactamase, that of *Enterobacter cloacae* P99. The effect is not seen with first-generation cephalosporins, since here deacylation generally seems faster than elimination of the leaving group, but it does clearly appear with cephamycins and third-generation cephalosporins. The structural and/or mechanistic features of the active site giving rise to this phenomenon may thus be common to all serine β -lactamases and transpeptidases.

INTRODUCTION

Previous work in this laboratory has demonstrated for class A β -lactamases [1,2] and for the DD-peptidase of *Streptomyces* R61 [3] that the presence of a leaving group X at the 3'-position of a cephem antibiotic (I) can lead to the generation of more-inert acyl-enzyme intermediates than from cephems lacking such a leaving group, and thus to β -lactamase inhibitors and, potentially, to better antibiotics. The inert acyl-enzymes were shown to arise by elimination of the 3'-leaving group (Scheme 1), yielding structure (III) (where EOH represents the enzyme and its nucleophilic serine hydroxy group at the active site). In the present paper we describe extension of this result to a class C β -lactamase, that of *Enterobacter cloacae* P99.

EXPERIMENTAL

Materials

The β -lactamase from *Enterobacter cloacae* P99 was purchased from the P.H.L.S. Centre for Applied

Microbiology and Research, Porton Down, Wilts., U.K., and used as supplied. It showed a specific activity against saturating cephalixin of 184 $\mu\text{mol}/\text{min}$ per mg (0.05 M-phosphate buffer, pH 7.0, at 30 °C), which favourably compares with a literature estimate [4]. Cephalothin, deacetylcephalothin, deacetoxycephalothin, keftet, cephaloridine, moxalactam and deacetoxycefotaxime (LY97964) were generously given by Eli Lilly and Co. Cefmenoxime, cefotaxime, ceftizoxime and cefoxitin were gifts from Abbott Laboratories, Hoechst-Roussel Pharmaceuticals, Smith, Kline and French Laboratories and Merck, Sharp and Dohme respectively. Thiophenol, which was distilled under vacuum before use, sodium 1-methyltetrazole-5-thiolate, $^2\text{H}_2\text{O}$ and $[\text{^2H}_4]\text{methanol}$ were purchased from Aldrich Chemical Co. Benzylpenicillin, 2,2'-dipyridyl disulphide and 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) were obtained from Sigma Chemical Co.

7 α -Methoxykeftet (see Tables, Table 2 in this case, for structures) was synthesized from cefoxitin and sodium 1-methyltetrazole-5-thiolate by the general procedure of

Scheme 1. β -Lactamase-catalysed hydrolysis of cephems

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Shimizu *et al.* [5,6]. The crude product was converted by diphenyldiazomethane treatment into the benzhydryl ester [2], which was purified by flash chromatography on silica gel (hexane/acetone, 11:9, v/v). Deprotection of the ester was achieved by trifluoroacetic acid treatment [2], and the resulting free acid was converted into its sodium salt with NaHCO_3 . Finally the salt was chromatographed in water on Sephadex G-10 and isolated by freeze-drying. It was characterized as the required product by its u.v. absorption [λ_{max} , (phosphate buffer, pH 7.0) 260 nm, $\epsilon = 6000 \text{ M}^{-1} \cdot \text{cm}^{-1}$] and ^1H n.m.r. [δ (p.p.m.) ($^2\text{H}_2\text{O}$) 3.30, 3.68 (2H, ABq, $J = 20 \text{ Hz}$, 2-*H*), 3.48 (3 H, s, OCH_3), 3.92 (2 H, s, Th- CH_2), 3.96, 4.20 (2 H, ABq, $J = 15 \text{ Hz}$, $-\text{CH}_2\text{S}-$), 3.99 (3 H, s, NCH_3), 5.08 (1 H, s, 6-*H*), 7.05 (2 H, m, Th-3'-*H* and Th-5'-*H*) and 7.34 (1 H, m, Th-4'-*H*)] spectra; the n.m.r. spectrum also indicated the absence of cefoxitin.

Decarbamoylcefloxitin, decarbamoyloxycefloxitin, thiophenoxycefloxitin, thiophenoxycephalothin and dansylcephalothin were available from previous investigations in this laboratory [2,6-8].

Analytical and kinetic methods

All kinetic experiments were performed at 30 °C in 0.1 M-phosphate buffer, pH 7.0. Absorption spectra and steady-state reaction rates were measured by means of a Cary 219 or Perkin-Elmer Lambda-4B spectrophotometer. β -Lactamase activity was routinely assayed against benzylpenicillin by the spectrophotometric method of Waley [9]. Pre-steady-state kinetics measurements were obtained from a Durrum D-110 stopped-flow spectrophotometer [7]. β -Lactamase concentrations were determined spectrophotometrically, by using a published absorption coefficient at 280 nm, $71000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [4]. Aqueous methanol solutions were prepared by making up weighed methanol to the required volumes with aqueous buffer.

Steady-state parameters were obtained by the method of Wilkinson [10] from initial-velocity measurements. The hydrolyses of cephalothin, cefoxitin, 7 α -methoxykefted, ceftizoxime, deacetoxycefotaxime, cefotaxime, cefmenoxime and moxalactam were monitored spectrophotometrically at 278 nm, 260 nm, 270 nm, 260 nm, 260 nm, 260 nm, 270 nm and 270 nm respectively in 1 cm-path-length cuvettes, and those of deacetylcephalothin, dansylcephalothin, cephaloridine, kefted, decarbamoylcefloxitin and decarbamoyloxycefloxitin were monitored at 275 nm, 270 nm, 270 nm, 290 nm, 280 nm and 280 nm respectively in 1 mm-path-length cuvettes. The reactions of thiophenoxycephalothin and thiophenoxycefloxitin were monitored at 412 nm in the presence of an excess of Ellman's reagent [6]. The steady-state rates of solvolysis of the various cepheims in methanol/water mixtures were determined spectrophotometrically in similar fashion and with the substrates at close-to-saturating ($\geq 10 K_m$) concentrations. The enzyme was stable in these solutions containing up to 3 M-methanol.

K_m values for cefoxitin, 7 α -methoxykefted, cefotaxime, cefmenoxime and moxalactam were determined as inhibition constants of cephalothin hydrolysis. The cephamycin and third-generation cephalosporin concentrations ranged between 0.1 and 10 μM .

The pre-steady-state phases of the reactions between the P99 β -lactamase (5–15 μM) and cefoxitin (10–50 μM), 7 α -methoxykefted (10–50 μM) and thiophenoxycefloxitin

(50 μM) were studied spectrophotometrically at 260 nm by the stopped-flow method. The reaction of thiophenoxycefloxitin was also monitored in the presence of Ellman's reagent at 412 nm. The resulting absorption changes were fitted to reaction schemes by the Simplex non-linear least-squares procedures described previously [7,11].

Detection of inert acyl-enzymes

Long-lived acyl-enzyme intermediates formed on reaction of the P99 β -lactamase with cefotaxime, cefmenoxime and moxalactam could be detected by dilution of a sample from an incubation mixture of enzyme (5 μM), saturated with cephalosporin (approx. 100 μM), into an assay cuvette containing 0.6 mM-benzylpenicillin. The initial rate of benzylpenicillin hydrolysis, measured spectrophotometrically, was close to zero but increased with time to a value representing full activity. The resulting absorbance changes were fitted to a first-order return of activity equation by a non-linear least-squares procedure [1].

Detection and determination of methanolysis products

^1H -n.m.r. spectroscopy was used to detect and determine the extent of acyl transfer to methanol when this co-solvent (as [$^2\text{H}_4$]methanol) was present in β -lactamase-catalysed solvolysis reaction mixtures in $^2\text{H}_2\text{O}$. Routinely, enzyme and substrate concentrations were about 5 μM and 10 mM respectively, the methanol concentration was about 2.5 M and 25 mM- NaHCO_3 was included as a buffer. Reactions were initiated by addition of enzyme and carried out at 30 °C. Typically, in the cases of substrates with 3'-leaving groups, two reaction products were seen in such reaction mixtures, one of which was the hydrolysis product (IV), whose spectrum has previously been reported for the first-generation cephalosporins and cephamycins [1,2,8], and which is readily recognized by the downfield resonances of the exomethylene hydrogen atoms at 5.63 and 5.66 p.p.m. in the case of the first-generation cephalosporins (IV, $\text{R}' = \text{H}$) and 5.65 and 5.69 p.p.m. with cephamycins (IV, $\text{R}' = \text{OMe}$). The third-generation cephalosporins cefotaxime and cefmenoxime yielded the same hydrolysis-product spectrum, analogous to those described above, with exomethylene resonances at 5.58 and 5.66 p.p.m. Methanolysis reaction mixtures contained a second product, whose n.m.r. spectrum was similar to that of the corresponding structure (IV), but with resonances in general shifted downfield. This was taken to be the methanolysis product (VI); spectra in $^2\text{H}_2\text{O}$ of the products of reactions carried out in methanol/water mixtures and isolated by freeze-drying showed methyl ester resonances in addition to those observed in the deuterated solvents. The characteristic exomethylene resonances of the methyl esters were at 5.66 and 5.72 p.p.m. (first-generation cephalosporins), 5.73 and 5.76 p.p.m. (cephamycins) and 5.62 and 5.70 p.p.m.

(VI)

(third-generation cephalosporins). The integration of these peaks allowed the fraction of the substrate methanolysed to be determined. Cephalosporins without leaving groups yielded product spectra characteristic of structure (V) [1,2,8] and those with the thiophenoxy leaving group, where elimination of the leaving group did not go to completion at the concentrations employed in these n.m.r. experiments, yielded spectra indicating the presence of structures (IV), (V) and (VI) [8].

RESULTS

Table 1 presents steady-state kinetic data for the P99- β -lactamase-catalysed hydrolysis of first-generation cephalosporins. There is no suggestion of a more-inert ($k_{\text{cat.}}$) or more tightly bound (K_m) intermediate in the cases where a leaving group is present, and, in particular, not in cephalothin and keftet, where very good leaving groups are present. There are three possible explanations of these results: (i) acylation is rate-determining in all cases and therefore the potential effects of elimination of the leaving group from structure (II) are not seen; (ii) deacylation is rate-determining but hydrolysis of structure (II) is faster than elimination of X from structure (II) for all X groups, i.e. $k_3 \gg k_3'$; (iii) deacylation is rate-determining and elimination of X does occur where X is a leaving group, but there is no leaving-group effect.

The kinetics of the P99- β -lactamase-catalysed solvolysis of cephalothin and keftet in methanol/water mixtures showed that $k_{\text{cat.}}$ increased linearly with methanol concentration (Fig. 1), and ^1H -n.m.r. spectra showed appropriate amounts of the methanolysis product (VI) ($R' = \text{OMe}$). These experiments suggest that with these cephalosporins, as in the hydrolysis of penicillins in the presence of this enzyme [12,13], deacylation is rate-determining; explanation (i) above therefore can not be correct.

The results with cephamycins and third-generation cephalosporins, described below, suggest that (iii) is not

Fig. 1. Rates of solvolysis of cephalothin (●) or keftet (○) in aqueous methanol solutions, catalysed by the *Enterobacter cloacae* P99 β -lactamase

The concentrations of cephalothin, keftet and enzyme were 4.9 mM, 1.9 mM and 0.046 μM respectively. Least-squares lines have been drawn through the points.

correct either, and thus (ii) must be. If (ii) were true, and if deacylation were rate-determining, as indicated above, all of the cephalosporins of Table 1, and specifically cephalothin and keftet, would yield different acyl-enzymes (II). That those generated by cephalothin and keftet are in fact different is confirmed by their significantly different $k_{\text{cat.}}$ values and by their different partitioning between water and methanol (the different slopes of the lines in Fig. 1). Hence it seems likely that the P99 β -lactamase catalyses the hydrolysis of structure (II), generated from first-generation cephalosporins, too effectively for the elimination of X to occur at the enzyme active site ($k_3 \gg k_3'$), and thus for any effect of leaving-group elimination to be seen.

The conclusion that $k_3 \gg k_3'$ for the pyridine and thiophenolate leaving groups is very reasonable in view

Table 1. Steady-state parameters for the P99- β -lactamase-catalysed hydrolysis of first-generation cephalosporins

Substrate	X	Leaving group	$k_{\text{cat.}}$ (s^{-1})*	K_m (mM)*
Cephalothin	OAc	✓	267	0.156
Cephaloridine	py ⁺	✓	663	2.7
Keftet	SHet [†]	✓	374	0.145
Thiophenoxycephalothin	SPh	— [‡]	227	0.925
Deacetoxycephalothin	—	—	300	1.4
Deacetylcephalothin	OH	— [‡]	355	0.325
Dansylcephalothin	NHDns	— [‡]	1990	0.418

* In 0.1 M-phosphate buffer, pH 7.0, at 30 °C.

[†] N-Methyltetrazolylthio.

[‡] These X groups are not eliminated before complete enzymic hydrolysis.

Table 2. Steady-state parameters for the P99- β -lactamase-catalysed hydrolysis of cephamycins

Substrate	X	Leaving group	$k_{\text{cat.}}$ (s^{-1})*	K_m (μM)*
Cefoxitin	CONH ₂	✓	0.31	1.9†
Methoxykeftel	SHet‡	✓	0.29	1.9†
Thiophenoxycefoxitin	SPh	—§	2.3	33
Decarbamoyloxycefoxitin	H	—	16.2	1700
Decarbamoylcefoxitin	OH	—§	11.0	250

* In 0.1 M-phosphate buffer, pH 7.0, at 30 °C.

† Determined by inhibition of cephalothin hydrolysis.

‡ *N*-Methyltetrazolylthio.

§ These X groups are not eliminated before complete enzymic hydrolysis.

of the rate constants for the elimination of these groups in free solution, 0.44 s^{-1} and 0.21 s^{-1} respectively [8,14]; from certain acyl-enzymes, at least, the elimination rates appear to be even smaller [6]. That this conclusion must now also be true for the acetate and *N*-methyltetrazolylthiolate leaving groups puts upper limits on k_3' for these groups ($k_3' < k_{\text{cat.}}$).

The cephamycins (I, $R' = \text{OMe}$), in contrast with the first-generation cephalosporins, show a clear indication of a leaving-group effect. Table 2 gives steady-state kinetic data for the P99- β -lactamase-catalysed hydrolysis of cephamycins, with and without leaving groups. Cephamycins with good leaving groups, cefoxitin and methoxykeftel, show a much lower $k_{\text{cat.}}$ and K_m , indicative of a more-inert intermediate, than do those without a leaving group, decarbamoyl and decarbamoyloxycefoxitin; furthermore, the $k_{\text{cat.}}$ values of cefoxitin and methoxykeftel are the same, within experimental uncertainty, indicative of a common (and thus, probably, a leaving-group-free) intermediate, such as structure (III). Steady-state kinetics experiments in methanol showed that rates of solvolysis of cefoxitin and 7 α -methoxykeftel increased linearly with the concentration of methanol, and to the same extent (results not shown), indicating rate-determining breakdown of a common intermediate.

Stopped-flow studies with cefoxitin and methoxykeftel show that the loss of the cephem chromophore occurs rapidly and completely (see, for example, the data shown in Fig. 2), and faster than turnover. This suggests an accumulating intermediate, and one of structure (III) rather than structure (II), since the chromophore is completely lost [1,2,8]. Quantitative interpretation of these data in terms of Scheme 1, and, employing the assumptions $k_3' \gg k_3$, $k_3' \gg k_2$ (and hence $k_4 = k_{\text{cat.}}$) and $k_2/K_s = k_{\text{cat.}}/K_m$, yielded (with $\Delta\epsilon_{\text{S} \rightarrow \text{P}} = \Delta\epsilon_{\text{E+S} \rightarrow \text{(III)}} = 7600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $6000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for cefoxitin and 7 α -methoxykeftel respectively) k_2 values for both cephamycins of approx. 50 s^{-1} and thus K_s values of approx. $300 \mu\text{M}$. These K_s values suggest that for decarbamoylcefoxitin and de-

Fig. 2. Absorption-coefficient changes at 260 nm on mixing cefoxitin (a) or methoxykeftel (b) with the *Enterobacter cloacae* P99 β -lactamase in the stopped-flow spectrophotometer

All concentrations after mixing were $13.0 \mu\text{M}$. The points are experimental and the lines were calculated as described in the text.

carbamoyloxycefoxitin, where the K_m values are comparable or larger, acylation is probably rate-determining; the $k_{\text{cat.}}$ values for the latter two compounds, somewhat smaller than the k_2 values for the former two, would seem reasonable for the rates of acylation by less chemically active substrates. This change of rate-determining

Table 3. Steady-state parameters for the P99- β -lactamase-catalysed hydrolysis of third-generation cephalosporins

Substrate	X	Leaving group	$k_{\text{cat.}}$ (s^{-1})*	K_m (μM)*
Cefotaxime	OAc	✓	0.051	0.14†
Cefmenoxime	SHet‡	✓	0.057	0.58†
Deacetoxycefotaxime	H	—	3.0	10.2
Ceftizoxime	3-H	—	1.4	8.1

* In 0.1 M-phosphate buffer, pH 7.0, at 30 °C.

† Determined by inhibition of cephalothin hydrolysis.

‡ *N*-Methyltetrazolylthio.

step would be analogous to that observed with the cephamycins and the RTEM-2 class A β -lactamase [2].

The steady-state kinetic parameters for thiophenoxy-cefoxitin lie intermediate between those of the above-mentioned substrates with 3'-leaving groups and those without. Stopped-flow studies, however, showed no burst loss of cephem chromophore, as seen for cefoxitin and methoxykeftel in Fig. 2, nor burst of thiophenol (in the presence of Ellman's reagent). Experiments in methanol/water solutions indicated that although significant amounts of methanolysis occurred (some 20% in 3 M-methanol) the steady-state rates under saturating conditions were not accelerated by methanol. These data suggest that acylation is rate-determining ($k_{\text{cat.}} = k_2$), $k_3 \gg k_3'$, and hence $k_3 \gg k_2$. That k_3 (which must be greater than 2.3 s^{-1}) is greater than k_3' is not surprising in view of the thiophenolate elimination rates discussed above. The rather strikingly smaller k_2 ($= k_{\text{cat.}}$) and K_s ($= K_m$) values may reflect specific and presumably hydrophobic interaction of the 3'-thiophenyl group with the enzyme. The P99 β -lactamase, and class C β -lactamases in general, are known to bind cephalosporins with positively charged 3'-substituents poorly [15] (note, for example, the K_m for cephaloridine in Table 1), which is also suggestive of interaction between these substituents and the enzyme. This may reflect a difference between class A β -lactamases, where there is little evidence of such interaction [1,2], and the class C enzymes.

The above interpretation of the results would indicate that the elimination of the 3'-leaving group of cephamycins retards deacylation of the acyl-enzymes generated with the P99 β -lactamase by at least 10-fold and probably significantly more than this.

Finally, there is also a leaving-group effect with the third-generation cephalosporins. Table 3 shows this in the steady-state kinetic parameters for hydrolysis of some of these compounds in the presence of the P99 β -lactamase. An intermediate (and probably, from the very similar $k_{\text{cat.}}$ values, the same one) accumulates in the case of cefotaxime and cefmenoxime, the substrates with leaving groups. This is shown by dilution of reaction mixtures into a benzylpenicillin assay medium [1]. The

enzyme activity, initially close to zero, increased with time, and eventually reached its original value before the cephalosporin addition, with first-order rate constants of 0.057 s^{-1} and 0.060 s^{-1} for cefotaxime and cefmenoxime respectively. These experiments strongly suggest that cefotaxime and cefmenoxime yield the same intermediate, presumably structure (III), whose hydrolysis is rate-determining at saturation. Deacetoxycefotaxime and ceftizoxime, the third-generation cephalosporins lacking a good 3'-leaving group, do not yield this species, as judged from their different and higher $k_{\text{cat.}}$ values. It is not known at present whether the $k_{\text{cat.}}$ values for deacetoxycefotaxime and ceftizoxime reflect acylation, k_2 , or deacylation, k_3 , but, irrespective of which it is, hydrolysis of the acyl-enzymes, presumably of structure (II), derived from these substrates must be much faster than those of structure (III) from cefotaxime and cefmenoxime.

Moxalactam, another third-generation cephalosporin, of structure (VII), has been shown to form an inert acyl-enzyme with class C β -lactamases [16–19]. From our measurements with the P99 enzyme we obtained $k_{\text{cat.}} = 3.3 \times 10^{-4} \text{ s}^{-1}$ and $K_m = 1.0 \mu\text{M}$ (derived from cephalothin inhibition) in 0.1 M-phosphate buffer, pH 7.0, at 30 °C. Inactivation of this enzyme with 1 equivalent of moxalactam followed by gel exclusion chromatography (1 cm $\times$ 20 cm column of Sephadex G-10) at 4 °C yielded in separate fractions a still-inactive enzyme and 1 equivalent of *N*-methyltetrazolylthiolate, the 3'-leaving group; the inert enzyme then regained activity at 30 °C with a rate constant equal to $k_{\text{cat.}}$. Thus it seems likely that the inert complex here too has the structure (III).

(VII)

DISCUSSION

The results described above strongly suggest that in cases where elimination of the 3'-leaving group is faster than hydrolysis of the initially formed acyl-enzyme (II) derived from reaction of cephem antibiotics with the class C β -lactamase of *Enterobacter cloacae* P99, a more-inert acyl-enzyme, of structure (III), forms (Scheme 1). The latter does not occur with first-generation cephalosporins, where hydrolysis of structure (II) is too rapid, but does more readily with cephamycins and third-generation cephalosporins. The ensuing decrease in both k_{cat} and K_m is at least 10-fold. This effect with third-generation cephalosporins has been previously noted in the literature, although its molecular basis has not yet been investigated. For example, cefotaxime has been shown to be a poorer β -lactamase substrate [20] and a better antibiotic [21] than desacetylcefotaxime. The structural and/or mechanistic features of the active site giving rise to this effect, which has now been demonstrated with class A [1,2] and C β -lactamases and with the DD-peptidase of *Streptomyces* R61 [3], may thus be common to all serine β -lactamases and transpeptidases [22].

The class C (or class I [23]) β -lactamases contribute significantly towards the resistance exhibited by Gram-negative bacteria towards β -lactam antibiotics [24–27]. Cephamycins and third-generation cephalosporins have been used to overcome this resistance because of their slow turnover by these enzymes. Nonetheless the affinity (K_m^{-1}) of these antibiotics for the class C enzymes is also high, which has led to discussion of the importance of a 'trapping' mechanism of resistance [28–32]. Although it is clear that many factors other than steady-state parameters for hydrolysis of the antibiotic will interact to dictate the effectiveness of antibiotic trapping, e.g. permeability of the outer membrane and the rate of biosynthesis of the β -lactamase [33], the present work indicates that one parameter that should favour the trapping of cephems rather than turnover should be the presence of a good 3'-leaving group.

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