Evidence for an oxyanion hole in serine β -lactamases and DD-peptidases

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A thionocephalosporin is shown to be a much poorer substrate of representative serine β -lactamases of class A (RTEM-2) and class C (*Enterobacter cloacae* P99) and a much poorer inhibitor of the *Streptomyces* R61 DD-peptidase than is the analogous oxo β -lactam. These results provide kinetic evidence for the existence of a catalytic oxyanion hole in these enzymes.

INTRODUCTION

Like serine proteinases, class A and C β -lactamases employ a nucleophilic serine hydroxy group and a double-displacement mechanism involving an acyl-enzyme intermediate in catalysing hydrolysis of their specific substrates [1–6]. A further important feature of the catalytic machinery of the serine proteinases is the oxyanion hole [7–9], which is believed to catalyse acyl transfer to and from the nucleophilic serine residue by stabilization of the tetrahedral intermediate and associated transition states. This stabilization is achieved through hydrogen-bond donation by specifically placed protein functional groups (H-A and H-B in structure I). Linear free-energy data from substrate perturbation studies support such electrophilic catalysis in the serine proteinases [10–12]. Although it does not seem that the catalytic machinery of β -lactamases can be identical with that of serine proteinases [13], an interesting question is whether the motif of the oxyanion hole has been, presumably independently, achieved by the former enzymes. The same question of course may be posed with respect to the bacterial cell-wall transpeptidases, which generally appear to be serine enzymes [13,14], and which may well be evolutionary precursors of the β -lactamases [15–17].

Thiono analogues of specific substrates of serine proteinases are, in general, poor or non-substrates, and this observation has been interpreted as evidence for the catalytic importance of the oxyanion hole to those enzymes [18,19]. The tetrahedral thiolate analogue of structure (I) is thought to be much less stabilized by the oxyanion hole because of the smaller tendency of S^- to accept hydrogen bonds and/or the difficulty of accommodating the longer (0.03–0.04 nm) C–S [20] (than C–O) in the hole, in a way that produces optimal hydrogen-

bonding and still maintains optimal binding of the rest of the substrate molecule.

We have used the thiono analogue probe for an oxyanion hole in the class A and class C β -lactamase active sites, employing the substrate pair (IIa) and (IIb). While this work was in progress, Herzberg & Moulton suggested, on the basis of the preliminary high-resolution crystal structure of the class A *Staphylococcus aureus* β -lactamase, that the serine β -lactamases do in fact make use of an oxyanion hole, which is comprised in this enzyme of protein backbone NH groups of Ser-70 and Gln-27 [21]. The presently reported kinetic studies with compounds (IIa) and (IIb) support this proposal, and there is the further indication that the D-alanyl-D-alanine transpeptidase/carboxypeptidase of *Streptomyces* R61 also employs an oxyanion hole in its interactions with β -lactam antibiotics.

EXPERIMENTAL

Materials

The β -lactamases of the RTEM-2 plasmid from *Escherichia coli* W3310 and of *Enterobacter cloacae* P99 were obtained from the Centre for Applied Microbiology and Research (Porton Down, Wilts., U.K.) and used as supplied. The soluble D-alanyl-D-alanine transpeptidase/carboxypeptidase (DD-peptidase) of *Streptomyces* R61 was generously given by Dr. J.-M. Ghuysen and Dr. J.-M. Frère (University of Liège, Liège, Belgium). Benzylpenicillin was purchased from Sigma Chemical Co. 7-Aminodesacetoxycephalosporanic acid was a gift from E. R. Squibb and Sons.

7-(Phenoxyacetamido)-3'-desacetoxycephalosporanic acid (IIa) was prepared by reaction of 7-amino-3'-desacetoxycephalosporanic acid with phenoxyacetyl chlor-

(IIa) X = O
(IIb) X = S

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ide (Aldrich Chemical Co.) in aq. NaHCO_3 solution, essentially as described by Chauvette *et al.* [22]. The product crystallized from ethyl acetate in 76% yield with m.p. 185 °C (lit. [22] m.p. 186–188 °C).

2,2,2-Trichloroethyl 7-(phenoxyacetamido)-3'-desacetoxycephalosporanate was also prepared by the method of Chauvette *et al.* [22]. The result crude material, an oil, was chromatographed on silica gel with toluene/ethyl acetate (4:1, v/v) and yielded 23 g (39%) of the pure Δ^3 isomer of the required product, identified by ^1H n.m.r. [δ (p.p.m.) (^2H)chloroform] 2.22 (3H, s, CH_3), 3.28 (1H, d, $J = 18$ Hz, $H-2$), 3.54 (1H, d, $J = 18$ Hz, $H-2$), 4.56 (2H, s, CH_2CO), 4.83 (1H, d, $J = 12$ Hz, CHCCl_3), 4.98 (1H, d, $J = 12$ Hz, CHCCl_3), 5.05 (1H, d, $J = 4.7$ Hz, $H-6$), 5.88, 5.89 (1H, dd, $J = 4.7$ and 9.1 Hz, $H-7$), 6.9–7.3 (5H, m, Ar) and 7.48 (1H, d, $J = 9.1$ Hz, NH) and m.p. 118 °C (lit. [22] m.p. 119 °C).

2,2,2-Trichloroethyl 7-(phenoxyacetamido)-8-thiono-3'-desacetoxycephalosporanate was prepared by a modification of the procedure of Wojtkowski *et al.* [23]. The 8-oxo analogue, prepared as described above (7.6 g, 15.8 mmol), and 3.8 g of boron sulphide (Alfa) were stirred in 50 ml of dry chloroform at 0 °C for 7 h. The solution was then heated under reflux until no starting material remained (approx. 2 h, followed by t.l.c.), cooled and filtered, and the solid was washed with dry chloroform. The solvent was evaporated from the combined chloroform solutions, and the resultant orange foam (7.6 g) was chromatographed on 300 g of silica gel with methylene chloride. The purest fractions containing the required product were combined, the solvent was evaporated, and the residue (1.1 g) was chromatographed on 50 g of silica gel with chloroform/benzene (5:1, v/v). The total yield of the pure thionolactam ester was 0.91 g (12%). Its ^1H n.m.r. spectrum [δ (p.p.m.) (^2H)chloroform] 2.19 (3H, s, CH_3), 3.25 (1H, d, $J = 18$ Hz, $H-2$), 3.53 (1H, d, $J = 18$ Hz, $H-2$), 4.60 (2H, s, CH_2CO), 4.76 (1H, d, $J = 12$ Hz, CHCCl_3), 5.11 (1H, d, $J = 12$ Hz, CHCCl_3), 5.48 (1H, d, $J = 4.3$ Hz, $H-6$), 5.64, 5.65 (1H, dd, $J = 4.3$ and 8.6 Hz, $H-7$), 7.0–7.4 (5H, m, Ar) and 7.27 (1H, d, $J = 7.9$ Hz, NH) and i.r. spectrum (absence of β -lactam $\text{C}=\text{O}$ and presence of $\text{C}=\text{S}$ stretching vibrations) agreed with those reported in the literature [23]. Also isolated from the second column were the 7-(phenoxythioacetamido)cephalosporin (30 mg, 0.4% yield) and the dithionated material (0.9 g, 11% yield).

The yields from this synthesis were not reproducible. The important variable appeared to be the boron sulphide; different lots from the vendor gave different yields, and more freshly prepared material (Noah Chemical Co.) and sonicated reaction mixtures [24] gave only degradation products. Other thionation reagents (e.g. Lawesson's reagent [25,26]) gave none of the required product. The situation with respect to the analogous penicillin, phenoxymethylthionopenicillin, was even less assured. We were able to obtain this compound only once [27] and even then not in sufficient quantity for detailed kinetic measurements.

Finally, 7-(phenoxyacetamido)-8-thiono-3'-desacetoxycephalosporanic acid (IIb) was obtained as a yellow solid in 65% yield on deprotection of the 2,2,2-trichloroethyl ester by zinc and acetic acid [23]. Fast-atom-bombardment m.s. yielded an $M+1$ peak (m/z 365) and a fragmentation pattern consistent with the required structure (IIb). The u.v.-absorption spectrum [λ_{max} (phosphate buffer) 307 nm, $\epsilon = 12\,300 \text{ M}^{-1} \cdot \text{cm}^{-1}$] agreed

with that reported [23] and its ^1H n.m.r. spectrum was as expected [δ (p.p.m.) ($^2\text{H}_2\text{O}$, HCO_3^-) 1.91 (3H, s, CH_3), 3.27 (1H, d, $J = 18$ Hz, $H-2$), 3.62 (1H, d, $J = 18$ Hz, $H-2$), 4.80 (2H, s, CH_2CO), 5.45 (1H, d, $J = 3.9$ Hz, $H-6,7$), 5.50 (1H, d, $J = 3.9$ Hz, $H-6,7$) and 7.0–7.4 (5H, m, Ar)].

Analytical and kinetic methods

Concentrations of the various enzymes were determined spectrophotometrically, by employing the absorption coefficients $29\,400 \text{ M}^{-1} \cdot \text{cm}^{-1}$, $71\,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $39\,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm for the RTEM-2 [28] and P99 [29] β -lactamases and the R61 DD-peptidase [30] respectively. Kinetic measurements with β -lactamases were made in 0.1 M-phosphate buffer, pH 7.0, at 30 °C. The reactions of compounds (IIa) and (IIb) were monitored spectrophotometrically at 265 nm ($\Delta\epsilon$ $6700 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and 307 nm ($\Delta\epsilon = 10\,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$) respectively, where the absorption coefficient changes were determined from absorption measurements after enzyme-catalysed hydrolyses. Steady-state kinetic parameters were obtained from initial-velocity measurements by the method of Wilkinson [31]. Routine assays of the β -lactamases and of the R61 DD-peptidase were performed spectrophotometrically with benzylpenicillin and hippuryl DL-phenyl-lactate [32] (Sigma Chemical Co.) respectively as substrates.

RESULTS AND DISCUSSION

The initial product of β -lactamase-catalysed hydrolysis of the cephalosporin (IIa) is, as confirmed by the ^1H n.m.r. spectrum [δ (p.p.m.) ($^2\text{H}_2\text{O}$, HCO_3^-) 1.87 (3H, s, CH_3), 3.13 (1H, d, $J = 17$ Hz, $H-2$), 3.41 (1H, d, $J = 17$ Hz, $H-2$), 4.55 (1H, d, $J = 4.8$ Hz, $H-6,7$), 4.68 (1H, d, $J = 4.8$ Hz, $H-6,7$), 4.71 (2H, s, CH_2CO) and 7.0–7.4 (5H, m, Ar)], the cephalosphoroate (IIIa). Although identification of the product of the RTEM-2 and P99 β -lactamase-catalysed hydrolyses of compound (IIb) was not possible because of the very low rates (see below), a ^1H n.m.r. spectrum of the product of hydrolysis of compound (IIb), catalysed by *Bacillus cereus* β -lactamase II, of which compound (IIb) is a relatively better substrate (B. P. Murphy & R. F. Pratt, unpublished work), was obtained. The close similarity between this n.m.r. spectrum [δ (p.p.m.) ($^2\text{H}_2\text{O}$, HCO_3^-) 1.87 (3H, s, CH_3), 3.12 (1H, d, $J = 18$ Hz, $H-2$), 3.39 (1H, d, $J = 18$ Hz, $H-2$), 4.63 (1H, d, $J = 4.8$ Hz, $H-6,7$), 4.69 (1H, d, $J = 4.8$ Hz, $H-6,7$), 4.71 (2H, s, CH_2CO) and 7.0–7.4 (5H, m, Ar)] and that of compound (IIa) indicates that compound (IIIb) is indeed the product of hydrolysis of compound (IIb) catalysed by the *Bacillus* enzyme. We assume that this is true too of the other β -lactamases. That the product of hydrolysis of compound (IIb) is not compound (IIIa) shows that H_2S is not eliminated in preference to the β -lactam nitrogen at the tetrahedral intermediate stage of the enzyme-catalysed reaction.

(IIIa) $X = \text{O}$

(IIIb) $X = \text{S}$

Table 1. Serine- β -lactamase-catalysed hydrolysis of the cephalosporins (IIa) and (IIb)

The kinetic measurements were made in 0.1 M-phosphate buffer, pH 7.0, at 30 °C.

Enzyme	Compound (IIa) (X = O)			Compound (IIb) (X = S)			$(k_{\text{cat.}}/K_m)_{\text{X-O}}$
	$k_{\text{cat.}}$ (s ⁻¹)	K_m (mM)	$k_{\text{cat.}}/K_m$ (s ⁻¹ ·M ⁻¹)	$k_{\text{cat.}}$ (s ⁻¹)	K_m (mM)	$k_{\text{cat.}}/K_m$ (s ⁻¹ ·M ⁻¹)	$(k_{\text{cat.}}/K_m)_{\text{X-S}}$
RTEM-2	4.2	0.24	1.8×10^4	2.2×10^{-4}	0.09	2.4	7.9×10^3
P99	1290	4.6	2.8×10^5	4.4×10^{-4}	6.0	0.073	3.8×10^6

In general, the susceptibility of thionoamides to nucleophilic attack by water in hydrolysis is very similar to that of amides under conditions of both acid and base catalysis [33,34]. Thionoamide hydrolysis is complicated by partitioning of tetrahedral intermediates between C-S and C-N bond fission [33,34]. Since we have not yet investigated the latter point with respect to the non-enzymic hydrolysis of compound (IIb), we do not report detailed kinetics of these reactions here, but our preliminary measurements of the rates of disappearance of compound (IIb) in HCl, KOH and phosphate buffer solutions suggest that the susceptibility of compound (IIb) towards nucleophilic cleavage by water is very similar to that of compound (IIa), i.e. compounds (IIa) and (IIb) are probably very similar with respect to their reactivity with oxygen nucleophiles.

The RTEM-2 and P99 β -lactamases both very weakly catalysed the disappearance and presumably hydrolysis of compound (IIb). They were not inactivated by it, since incubation of compound (IIb) (1.3 mM) with these enzymes led to no loss of β -lactamase activity; there is thus no evidence for accumulation of a slowly hydrolysing acyl-enzyme. Steady-state parameters for the RTEM-2 and P99 β -lactamase-catalysed hydrolysis of compound (IIb) and of compound (IIa) for comparison are reported in Table 1. The very plain conclusion, from comparison of the $k_{\text{cat.}}/K_m$ values, is that compound (IIb) is a much poorer substrate of both enzymes than is compound (IIa). Preliminary observations suggested that a similar difference existed between the reactivity of phenoxy-methylpenicillin and phenoxymethylthionopenicillin with the class A β -lactamase I of *Bacillus cereus* and the RTEM-2 enzyme [27]. This difference, caused by the substitution of a sulphur atom for an oxygen atom, is of the same order as, or greater than, the difference observed with chymotrypsin and subtilisin [18,19], and, as in these cases, can be interpreted as good evidence for the existence of a catalytic oxyanion hole.

It is evident also from Table 1 that the major effect of the oxygen-to-sulphur transformation is on $k_{\text{cat.}}$, which would be in accord with the participation of the oxyanion hole in catalysis. In contrast, binding before catalysis (K_m) appears to be much less affected by the substrate perturbation, and in the case of the RTEM-2 enzyme this parameter is actually more favourable for catalysis in compound (IIb) than in compound (IIa). In principle, the latter result could be interpreted, following Campbell *et al.* [18], in terms of differential hydrogen-bonding in the case of this enzyme, i.e. there is loss of hydrogen-bonding to the β -lactam carbonyl oxygen atom in the Michaelis complex. Any rigorous interpretations along these lines, however, must at present assume either that acylation is rate-determining in all cases, which is unlikely

with compound (IIa) and the P99 enzyme at least [35], or, probably more likely, that the effect of the oxygen-to-sulphur transformation is equally present in both acylation and deacylation.

Like other cephalosporins, compound (IIa) readily inactivates the *Streptomyces* R61 transpeptidase. For example, 181 μM -compound (IIa) completely inactivated the R61 enzyme (5.49 μM) in 20 mM-phosphate buffer at 37 °C, with a half-time of about 8.0 min. At the same concentration and under the same conditions compound (IIb) had no effect on the activity of the enzyme over a 1.5 h time span. A combination of compound (IIa) (181 μM) and compound (IIb) (406 μM) inactivated the enzyme significantly more slowly (about 8-fold) than did compound (IIa) alone. These results suggest that although compound (IIb) probably binds at the active site, and somewhat more tightly than does compound (IIa) itself, it is a much poorer acylating reagent for the active-site serine residue of the R61 DD-peptidase [14] than is compound (IIa), and thus that an oxyanion hole is also present in this enzyme and participates in its inactivation by β -lactam antibiotics.

In conclusion, these experiments point once more [15–17] to the similarities between the interactions of β -lactamases and DD-peptidases with β -lactam antibiotics. There must be differences, of course [17,32], but an oxyanion hole may well be a conserved feature that these two groups of serine enzymes have in common. The same electrophilic site would quite probably catalyse the reactions of acyclic substrates also.

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