Single-turnover and steady-state kinetics of hydrolysis of cephalosporins by β -lactamase I from *Bacillus cereus*

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The kinetics of the hydrolysis of two cephalosporins by β -lactamase I from *Bacillus cereus* 569/H/9 has been studied by single-turnover and steady-state methods. Single-turnover kinetics could be measured over the time scale of minutes when cephalosporin C was the substrate. The other substrate, 7-(2',4'-dinitrophenylamino)deacetoxycephalosporanic acid, was hydrolysed even more slowly, and has potential for use in crystallographic studies of β -lactamases. Comparison of single-turnover and steady-state kinetics showed that, for both substrates, opening the β -lactam ring (i.e. acylation of the enzyme) was the rate-determining step. Thus the non-covalent enzyme–substrate complex is expected to be the intermediate observed crystallographically.

INTRODUCTION

β -Lactamases are efficient and clinically important enzymes that play an important part in antibiotic resistance. Mechanistically, as far as we know, β -lactamases are either 'serine' enzymes or 'zinc' enzymes. These are convenient short-hand descriptions which summarize the conclusions from many investigations. At least two, rather dissimilar, β -lactamases, require zinc ions for activity (Sabath & Abraham, 1966; Saino *et al.*, 1982; Bicknell *et al.*, 1985). Detailed studies on one of these enzymes (β -lactamase II from *Bacillus cereus*) at low temperatures points to non-covalent catalysis, with the metal ion playing an intimate role in that catalysis (Bicknell & Waley, 1985). The β -lactamases that are serine enzymes function by covalent catalysis; the key intermediate is an acyl-enzyme (Knott-Hunziker *et al.*, 1979, 1982; Cohen & Pratt, 1980; Fisher *et al.*, 1980, 1981; Dalbadie-McFarland *et al.*, 1982; Sigal *et al.*, 1982, 1984; Cartwright & Coulson, 1980; Frère, 1981; Cartwright & Fink, 1982; Joris *et al.*, 1984; Anderson & Pratt, 1981, 1983). Thus the hydrolysis of a penicillin proceeds by way of an acyl-enzyme that is an α -penicilloyl ester of a serine residue (serine-70 or serine-80 in class A or class C β -lactamases respectively; Ambler, 1980; Jaurin & Grundström, 1981). The first stage in our understanding of β -lactamase mechanisms, a knowledge of the main type of intermediate, has thus been reached for several β -lactamases. One aspect of the next stage is the timing of events. The present paper is concerned with this aspect, namely whether acylation or deacylation is rate-determining. Understanding of structure–activity relationship demands knowledge of the rate-determining step. A rather direct way of finding which is the rate-determining step is to compare single-turnover and steady-state kinetics. The former gives the rate of opening the β -lactam ring, measured spectroscopically. The latter gives an overall rate for acylation and deacylation but does not permit their relative contribution to the overall rate to be determined.

The use of slow substrates in mechanistic studies has both advantages and drawbacks. The obvious drawback is that the mechanism could be atypical. The advantages are that pre-steady-state kinetics can be studied with ordinary apparatus and, especially, that crystallography (at ordinary temperatures) demands slow substrates. Crystallographic studies have been in progress on β -lactamases from *Escherichia coli* (RTEM) (Knox *et al.*, 1976), *Bacillus cereus* (β -lactamase I) (Aschaffenburg *et al.*, 1978), *Enterobacter cloacae* (P99) (Charlier *et al.*, 1983), *Staphylococcus aureus* (PC1) (Moult *et al.*, 1985) and *Bacillus licheniformis* (749/C) (Dideberg *et al.*, 1985). β -Lactamase I hydrolyses cephalosporins slowly (Abraham & Newton, 1956; Crompton *et al.*, 1962; Kuwabara & Abraham, 1967; Abraham & Waley, 1979), and the present paper describes the kinetics of hydrolysis of two cephalosporins by β -lactamase I. One of these, Dnp-ADCA, seems well suited for crystallography. It offers the hope of studying a productive enzyme–substrate complex, particularly useful when the enzyme catalyses an effectively irreversible reaction.

MATERIALS AND METHODS

Materials

β -Lactamase I from *Bacillus cereus* 569/H/9 was prepared as described previously (Davies *et al.*, 1974; Baldwin *et al.*, 1980). Cephalosporin C, a gift from Glaxo Laboratories, Greenford, Middx., U.K., was recrystallized from aqueous acetone before use. Dnp-ADCA (I) was

Abbreviations used: Dnp-ADCA, 7-(2',4'-dinitrophenylamino)deacetoxycephalosporanic acid; 7-ADCA, 7-aminodeacetoxycephalosporanic acid; FDNB, 1-fluoro-2,4-dinitrobenzene; Dns, 5-dimethylaminonaphthalene-1-sulphonyl.

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Fig. 1. Absorption spectra of Dnp-ADCA before and after hydrolysis

Dnp-ADCA was 0.1 mM in pH 6 buffer (0.2 M-succinic acid/sodium succinate). Hydrolysis was effected by the β -lactamase (Berks *et al.*, 1982) from *Pseudomonas aeruginosa*. The isosbestic points at 298 and 350 nm were sharp. The Dnp-ADCA had λ_{max} at 350 nm and ϵ 13.4 mM⁻¹·cm⁻¹; the hydrolysis product had λ_{max} at 357 nm and ϵ 13.7 mM⁻¹·cm⁻¹.

prepared from 7-ADCA, a gift from Eli Lilly and Co., Indianapolis, IN, U.S.A., as follows.

FDNB (0.948 g, 5.04 mmol) in acetone (2 ml) was added over 30 min to 7-ADCA (1.11 g, 4.2 mmol) in 3% (w/v) NaHCO₃ (45 ml)/acetone (30 ml). After 24 h, t.l.c. [acetone/acetic acid (19:1, v/v); silica plate] showed the presence of FDNB (yellow, R_F 0.93), 2,4-dinitrophenol (yellow, R_F 0.86), Dnp-ADCA (yellow, R_F 0.72) and unchanged 7-ADCA (detected with I₂ vapour, R_F 0.5).

A second portion of FDNB (0.84 mmol in 0.5 ml of acetone) was then added and the mixture was left at room temperature for a further 24 h; t.l.c. showed some hydrolysed (β -lactam ring opened) 7-Dnp-ADCA (yellow, R_F 0.05). A maximum quantity of the desired product was present after 48 h as judged by t.l.c.

Acetone was removed by evaporation and the reaction mixture extracted with diethyl ether (3 × 50 ml) to remove excess FDNB, acidified to pH 4.0, and extracted with ethyl acetate (3 × 50 ml). The ethyl acetate extracts were extracted with 3% (w/v) NaHCO₃, then adjusted to pH 3.0 and extracted with ethyl acetate. The required product and 2,4-dinitrophenol were present. The cephalosporin was separated from the dinitrophenol by precipitation with *NN'*-dibenzylethylenediamine diacetate (in chloroform). The salt was converted into the potassium salt (yield 28%) via the free acid. The concentration of solutions was determined from ϵ 13400 M⁻¹·cm⁻¹ at 350 nm (Fig. 1). The absorption coefficient was determined after gel filtration of a sample on a column of Sephadex G-10, freeze-drying pure Dnp-ADCA and recording the absorbance of a solution of known molarity (by weight). P.m.r. of sodium salt: δ (p.p.m. from tetramethylsilane (2H₂O) 1.8 (3H, s, 3'-CH₃), 3.1, d, and 3.45, d, CH₂, 5.2 (1H, d), 5.5 (1H, d)

(C₆-H, C₇-H), and 8.95 (1H, d), 8.2 (1H, double doublet), 7.1 (1H, d) from the protons in the dinitrophenyl ring at C-3, C-5 and C-6 respectively. The i.r. (paraffin mull) showed the characteristic β -lactam absorbance at 1758 cm⁻¹. The fluorescence spectrum in aqueous solution at pH 7 had λ_{max} (emission) = 420 nm with excitation at 350 nm. After alkaline hydrolysis of the Dnp-ADCA with 0.1 M-NaOH, or hydrolysis by the β -lactamase from *Pseudomonas aeruginosa* (Berks *et al.*, 1982), the spectrum changed as shown in Fig. 1; the clean isosbestic point was consistent with the formation of a single hydrolysis product. On enzymic hydrolysis at pH 7, one proton was liberated; no change in fluorescence on hydrolysis was detected. Hydrolysis of the Dnp-ADCA by β -lactamase I was measured at 450 nm; at pH 6 and 0 °C, $\Delta\epsilon$ = 0.663 mM⁻¹·cm⁻¹.

Methods

Assays were carried out on a Cary model 219 spectrophotometer with a pathlength of 1–10 mm. With the exception of those assays in which (NH₄)₂SO₄ was present, the following buffers were used (all in 0.5 M-NaCl 1 mM-EDTA); 0.05 M-acetic acid/sodium acetate, pH 4.5; 0.2 M-succinic acid/sodium succinate, pH 6; 0.05 M-phosphoric acid/sodium phosphate, pH 7; 0.06 M-NaHCO₃/Na₂CO₃, pH 10. Buffers containing (NH₄)₂SO₄ were prepared in 0.2 M-succinic acid/sodium succinate/1 mM-EDTA and adjusted to pH 6 after the addition of the (NH₄)₂SO₄.

Steady-state kinetic parameters were determined either from initial rates with the 'chord' method (Waley, 1981) and a median program of Dr. A. Cornish-Bowden for a direct linear plot based on that described by Cornish-Bowden & Eisenthal (1978), or from progress curves and a half-time analysis (Wharton & Szawelski, 1982); in the latter case, the stability of the enzyme over the course of the assay was confirmed by Selwyn's (1965) method. The linearity of half-time plots from complete progress curves and of half-reciprocal plots of initial rates were taken as evidence for Michaelis-Menten kinetics. Inhibition by product was not observed. Assays with cephalosporin C were carried out at 260 nm, and those with compound (I) at 450 nm.

Rate constants for single-turnover assays were obtained from fitting the first-order equation:

$$p = p_{\infty} (1 - e^{-k_{\text{obs}} \cdot t})$$

where p is the concentration of product at time t , and p_{∞} is the final concentration of product. The method used was as follows. The sum (Q) of the squares of the residuals for N points is:

$$Q = \sum_{i=1}^N [p_i - p_{\infty} (1 - e^{-k_{\text{obs}} \cdot t_i})]^2$$

Differentiating with respect to the parameters p_{∞} and k_{obs} , and equating the differentials to zero, gives:

$$\sum_i p_i \cdot (1 - e^{-k_{\text{obs}} \cdot t_i}) - p_{\infty} \cdot \sum_i (1 - e^{-k_{\text{obs}} \cdot t_i})^2 = 0 \quad (1)$$

$$\sum_i p_i \cdot e^{-k_{\text{obs}} \cdot t_i} - p_{\infty} \cdot \sum_i e^{-k_{\text{obs}} \cdot t_i} + p_{\infty} \cdot \sum_i e^{-2k_{\text{obs}} \cdot t_i} = 0 \quad (2)$$

The value of p_{∞} from eqn. (1) was substituted into eqn. (2) and the non-linear equation for k_{obs} was then solved numerically; the bisection method (e.g. Conte & de Boor, 1980) was used, in a BASIC program implemented on a PDP11 or Apple IIe computer.

Table 1. Kinetic parameters for the hydrolysis of Dnp-ADCA (I) and cephalosporin C by β -lactamase I

The experiments were carried out, as described in the text, at 0.4 °C; the standard errors are given.

Substrate	$k_{\text{cat.}}$ (min^{-1})	K_m (mM)	$k_{\text{cat.}}/K_m$ ($\text{mM}^{-1} \cdot \text{min}^{-1}$)	k_{+2} (min^{-1})	K_s (mM)	k_{+2}/K_s ($\text{mM}^{-1} \cdot \text{min}^{-1}$)
(a) Dnp ADCA	0.0068 ± 0.0005	1.7 ± 0.6	0.004 ± 0.0006	0.0072 ± 0.0005	2.0 ± 0.2	0.0036 ± 0.0004
(b) Cephalosporin C						
At pH 7	0.9 ± 0.005	0.16 ± 0.03	5.6 ± 0.3	1.2 ± 0.1	0.08 ± 0.02	15 ± 2
At pH 4.5	0.76 ± 0.014	2.3 ± 0.08	0.33 ± 0.02	*	*	0.38 ± 0.02
At pH 10	0.35 ± 0.008	0.82 ± 0.02	0.43 ± 0.02	*	*	0.5 ± 0.2

* Only the ratio, k_{+2}/K_s could be determined (see the text).

RESULTS AND DISCUSSION

Steady-state kinetics

Cephalosporin C is slowly hydrolysed by β -lactamase I (Abraham & Newton, 1956; Crompton *et al.*, 1962); the specificity constant ($K_{\text{cat.}}/K_m$) was $5.6 \text{ mM}^{-1} \cdot \text{min}^{-1}$ (Table 1). Dnp-ADCA (I) was hydrolysed even more slowly, by a factor of about 1000 (Table 1). Such very low rates of hydrolysis are required for substrates that are to be useful in crystallographic studies. However, it is important to check that specific enzymic catalysis is occurring. Initial-rate measurements of the hydrolysis of Dnp-ADCA followed Michaelis-Menten kinetics at 0.4 °C (Fig. 2). Selwyn's (1965) test showed that β -lactamase I was stable during catalysis at 0.4 °C, but not at temperatures above 20 °C. β -Lactamase I that had been specifically inactivated by 6 β -bromopenicillanic acid no longer catalysed the hydrolysis of Dnp-ADCA. We concluded that Dnp-ADCA was a true substrate of

β -lactamase I. When the initial concentration of substrate was ten times K_m , zero-order kinetics were observed from the start of the reaction; no burst was observed, although the concentration of enzyme (0.6 mM) was high enough to see one. Hysteresis in β -lactamase I action is apparently restricted to penicillins with bulky side chains (Citri *et al.*, 1976). A 'lag test' was carried out as follows. A portion from the reaction mixture during the hydrolysis of Dnp-ADCA (at an initial concentration ten times the K_m) by β -lactamase I was diluted into a solution of 20 mM-benzylpenicillin in the pH-stat (at pH 8, 2 °C) and the rate of hydrolysis of the benzylpenicillin measured. There was no decrease in activity relative to that of the free enzyme. This absence of a lag may be contrasted with the results of comparable experiments in which cloxacillin was hydrolysed by the β -lactamase from *Pseudomonas aeruginosa* (Knott-Hunziker *et al.*, 1982), or cefoxitin was hydrolysed by the RTEM β -lactamase from *Escherichia coli* (Fisher *et al.*, 1980a). In both the cited experiments a lag was observed and shown to be due to the accumulation of an acyl-enzyme. Thus the experiment reported here suggests that the acyl-enzyme does not accumulate during the hydrolysis of Dnp-ADCA by β -lactamase I.

Fig. 2. Variation of initial rate of hydrolysis of Dnp-ADCA by β -lactamase I with substrate concentration

The hydrolysis at pH 6 and 0.4 °C was measured spectroscopically. This is a half-reciprocal plot of the initial-rate data.

Difference spectra during turnover

Difference spectra were recorded on incubating Dnp-ADCA (2 mM) with β -lactamase I (0.4 mM) at 0 °C, measurements being read against a cuvette containing an equivalent concentration of substrate. A 0.1 cm pathlength was used because of the high absorbance. The autobaseline facility on the Cary 219 instrument was employed to obtain a horizontal baseline in these experiments. Repetitive scanning over the wavelength range 345–500 nm indicated the appearance of product at a rate consistent with hydrolysis of β -lactamase I, but no detectable intermediate. The isosbestic point at 350 nm was clean (within an absorbance of 0.01), indicating the absence of species with spectra differing markedly from those of substrate or product (Fig. 1; for the sake of clarity, only the initial and final spectra are shown). The control difference spectrum of product against substrate was obtained by hydrolysing Dnp-ADCA under identical conditions with the class-C β -lactamase from *Pseudomonas aeruginosa*, which hydrolyses it much more rapidly than does β -lactamase I.

Fig. 3. Progress curve for the hydrolysis of Dnp-ADCA (I) under single-turnover conditions

The concentrations of enzyme and substrate were 1.2 mM and 0.06 mM respectively; the reaction was carried out at pH 6 and 0.4 °C.

Single-turnover experiments

The simplest acyl-enzyme mechanism is:

Values of k_{+2} and K_s may be obtained from experiments in which the concentration of enzyme (e_0) is appreciably greater than the concentration of substrate (s_0), since the observed spectral change in the hydrolysis of the Dnp-ADCA is associated with opening the β -lactam ring. When $e_0 > 5 \times s_0$ there was an exponential progress curve (Fig. 3). The observed first-order rate constant, $k_{\text{obs.}}$, is given (Bender & Zerner, 1962; Hollaway *et al.*, 1969; Laidler & Bunting, 1973) by:

$$k_{\text{obs.}} = \frac{k_{+2} \cdot e_0}{e_0 + K_s}$$

The results for the hydrolysis of Dnp-ADCA (Table 1) showed that k_{+2} was equal (within the experimental error) to the steady-state $k_{\text{cat.}}$. Thus acylation was the rate-determining step. The intermediate that accumulated was the non-covalent enzyme-substrate complex, and $K_s = K_m$.

Single-turnover experiments on the hydrolysis of cephalosporin C by β -lactamase I at pH 7 were analysed similarly (Fig. 4a). Again, k_{+2} was, effectively, the same as $k_{\text{cat.}}$ (Table 1) and so acylation is rate-determining. We could not tell whether the same was true at pH 4.5, because $k_{\text{obs.}}$ was directly proportional to e_0 (Fig. 4b). The slope of the line gave k_{+2}/K_s , which was in fair agreement with the value of $k_{\text{cat.}}/K_m$ found from steady-state experiments (Table 1). The same was true at pH 10 (Table 1). This result is demanded by most (but not all) mechanisms.

Effect of $(\text{NH}_4)_2\text{SO}_4$ on hydrolysis of Dnp-ADCA by β -lactamase I

The exceptionally slow turnover of Dnp-ADCA by β -lactamase I suggested that it might be used for an X-ray-diffraction study of the enzyme-substrate complex. Since β -lactamase I is crystallized from $(\text{NH}_4)_2\text{SO}_4$ (Aschaffenburg *et al.*, 1978), the effects of high

Fig. 4. Single-turnover experiments with β -lactamase I and cephalosporin C

(a) Variation of rate of hydrolysis of cephalosporin C by β -lactamase I with enzyme concentration at pH 7. The reaction was measured spectroscopically and was carried out at 0.4 °C. (b) Variation of rate constant with concentration of enzyme at pH 4.5. A plot of $k_{\text{obs.}}$ (from a single-turnover assay) against the enzyme concentration for the hydrolysis of cephalosporin C by β -lactamase I at 0.4 °C is shown.

concentrations of the latter on the steady-state kinetic parameters were measured. The value of $k_{\text{cat.}}$ was unchanged, but K_m decreased, as did the solubility of the substrate (Table 2). These results lent support to the hope that there would be adequate occupancy of the active sites in the crystal. The half-life was about 2 h at 0 °C and was estimated to be about 6 h – 12 °C. It was not possible to measure enzymic hydrolysis at concentrations of $(\text{NH}_4)_2\text{SO}_4$ greater than 35% saturation; the enzyme was insufficiently soluble and, moreover, the non-enzymic reaction of Dnp-ADCA with NH_3 made the analysis of progress curves unreliable. Whether this non-enzymic reaction will be significant in crystallographic experiments is unclear.

Acyl-enzymes as intermediates in β -lactamase action

Although the first evidence for acyl-enzymes came from the use of inhibitors, there have now been several studies in which turnover of substrates has been

Table 2. Effect of $(\text{NH}_4)_2\text{SO}_4$ on the hydrolysis of Dnp-ADCA by β -lactamase I

Progress curves were analysed to obtain the values of the steady-state kinetic parameters at pH 6 and 0.4 °C. The last entry refers to an experiment with 5 M-NaCl at pH 6 and 12 °C.

Concn. of $(\text{NH}_4)_2\text{SO}_4$ (% satn.)	$10^3 \times k_{\text{cat.}}$ (min^{-1})	K_m (mM)	Solubility of substrate (mM)
0	6.8	1.7	25
10	6.7	1.2	
20	7.2	0.82	
35	7.15	0.59	2.8
70			0.5
5 M-NaCl	14.2	0.67	6.0

Table 3. Isolation of acyl-enzymes from serine β -lactamases

β -Lactamase	Substrate	Reference	Category
RTEM*	Cefoxitin	Fisher <i>et al.</i> (1980)	Covalently modified enzyme was isolated and kinetics used to show that it was a true intermediate
PC1†	Dansyl-cephalosporin‡	Anderson & Pratt (1983)	
<i>Ps. aeruginosa</i>	Cloxacillin	Knott-Hunziker <i>et al.</i> (1982)	
β -Lactamase I	Dansyl-penicillin	Cartwright & Fink (1982)	Covalently modified enzyme was isolated but not shown to be an intermediate
β -Lactamase P99	Cloxacillin	Joris <i>et al.</i> (1984)	
	Methicillin or cloxacillin	Kiener <i>et al.</i> (1980)	
PC1†	Dansyl-cephalexin	Anderson & Pratt (1983)	Covalently modified enzyme did not accumulate
β -Lactamase I	Cephalosporin C or Dnp-ADCA	The present paper	

* Plasmid-encoded β -lactamase from *E. coli*.

† *Staphylococcus aureus* PC1 β -lactamase.

‡ Sodium 3-dansylamidomethyl-7 β -(thien-2'-yl)acetamidoceph-3-em-4-oate.

interrupted and intermediates were isolated (Table 3). There have also been experiments (Dns-cephalexin and β -lactamase PC1; Anderson & Pratt, 1983) in which, as in the results reported here, there is no accumulation of acyl-enzyme. Fisher (1984) has suggested that, for good substrates and class-A β -lactamases, acylation is rate-determining. On the other hand, even for good substrates, deacylation is rate-determining for class-C β -lactamases. Finally, for class-B β -lactamases, the evidence is against acyl-enzyme intermediates (Bicknell & Waley, 1985). It is clear that more work is required to see if these generalizations hold true when more examples are studied.

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