Substrate-induced inactivation of the OXA2 β -lactamase

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The hydrolysis time courses of 22 β -lactam antibiotics by the class D OXA2 β -lactamase were studied. Among these, only three appeared to correspond to the integrated Henri–Michaelis equation. ‘Burst’ kinetics, implying branched pathways, were observed with most penicillins, cephalosporins and with flomoxef and imipenem. Kinetic parameters characteristic of the different phases of the hydrolysis were determined for some substrates.

Mechanisms generally accepted to explain such reversible partial inactivations involving branches at either the free enzyme or the acyl-enzyme were inadequate to explain the enzyme behaviour. The hydrolysis of imipenem was characterized by the occurrence of two ‘bursts’, and that of nitrocefin by a partial substrate-induced inactivation complicated by a competitive inhibition by the hydrolysis product.

INTRODUCTION

Two groups of β -lactamases can be distinguished. The metallo-proteins of class B (Ambler, 1980), which contain at least one Zn^{2+} ion per active subunit, are characterized by a rather broad spectrum against β -lactam antibiotics, with the remarkable exception of the *Aeromonas hydrophila* enzyme, which efficiently hydrolyses only imipenem and ampicillin (Felici et al., 1993). The kinetic homogeneity of class C ‘cephalosporinases’ (Galleni and Frère, 1988; Galleni et al., 1988) contrasts with the diversity of the catalytic properties of class A β -lactamases (Matagne et al., 1990). Class D enzymes consistently hydrolyse oxacillin and cloxacillin faster than benzylpenicillin, justifying their designation as ‘oxacillinases’ (Dale and Smith, 1971, 1972, 1974; Matthew et al., 1979; Medeiros et al., 1985). However, the most unexpected result concerning this latter class is the finding that, with at least the OXA2 β -lactamase, ‘burst’ kinetics prevail with many substrates (Ledent et al., 1993). This kind of behaviour was also described for the hydrolyses of cefoxitin by the TEM1 and TEM2 β -lactamases (Fisher et al., 1980; Citri and Zyk, 1982; Citri et al., 1984; Faraci and Pratt, 1986), of quinacillin by the penicillinase from *Staphylococcus aureus* (Virden et al., 1978) and of pyridine-2-azo-4’-(*N,N*’-dimethylalanine)cephalosporin by that from *Enterobacter cloacae* P99 (Cartwright and Waley, 1987). Such biphasic hydrolyses reflect a slow conformational transition of the protein induced by the substrate (Citri et al., 1976; Kiener and Waley, 1977; Klemes and Citri, 1979, 1980) or/and a progressive accumulation of a more stable acyl-enzyme after the departure of the 3’-leaving group of cephalosporins and cephamycins (Faraci and Pratt, 1985; Mazzella and Pratt, 1989). These ‘bursts’ are generally explained by a reaction pathway (Scheme 2 below) in which these two possible modifications occur at the level of the acyl-enzyme (Fisher et al., 1978; Kiener et al., 1980; Frère et al., 1982a,b; Persaud et al., 1986; Fink et al., 1987). Alternatives in which the partition takes place at the stage of free enzyme or of the Henri–Michaelis complex were discussed by Waley (1991) and mechanisms containing branches at more than one species by Virden et al. (1978) and Frère (1981).

The present investigation was initiated to obtain detailed data about the catalytic properties of the OXA2 β -lactamase, and in particular about the mechanism of partial inactivation induced by a large majority of substrates.

MATERIALS AND METHODS

Antibiotics

Ampicillin and oxacillin were from Bristol–Myers Squibb Belgium (Brussels, Belgium), 6-aminopenicillanic acid, carbenicillin, cloxacillin, dicloxacillin and methicillin were from Beecham Research Laboratories (Brentford, Middx., U.K.), benzylpenicillin was from Rhône-Poulenc (Paris, France), cefaclor, cefamandole, cefazolin, cephaloglycin, cephaloridine, cephalosporin C and cephalothin were from Eli Lilly and Co. (Indianapolis, IN, U.S.A.), ceftazidime and cefuroxime were from Glaxo Group Research (Greenford, Middx., U.K.), cephacetrile was from Ciba–Geigy (Basel, Switzerland), flomoxef was from Shionogi Research Laboratories (Osaka, Japan), and imipenem was from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). All these compounds were kindly given by the respective companies. Nitrocefin was purchased from Oxoid (Basingstoke, Hants., U.K.) and 7-aminocephalosporanic acid from Janssen Pharmaceutica (Beerse, Belgium). The structures of the compounds which are not shown in Matagne et al. (1990) are displayed in Figure 1.

Proteins

The OXA2 β -lactamase was produced and purified as described by Ledent et al. (1993). The preparation of the Zn^{2+} - β -lactamase of *Bacillus cereus* 5/B/6 was that described by De Meester et al. (1987).

Spectrophotometers

All the spectrophotometric measurements were performed with the help of a UVIKON 860 (Kontron Instruments) spectrophotometer interfaced to a Copam⁺ PC 88C microcomputer. Readings to six decimal places were transferred to the microcomputer, where all the arithmetic operations were performed without decreasing that number. A Biologic SFM-3 stopped-flow instrument was utilized for monitoring rapid reactions.

Kinetic measurements

Complete time-courses of the hydrolysis of the substrate were usually recorded at 482 nm for nitrocefin, 273 nm for cephalothin,

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Figure 1 Structures of dicloxacillin, cefuroxime, flomoxef, imipenem and cefaclor

260 nm for the other cephalosporins, 270 nm for flomoxef, 235 nm for 6-aminopenicillanic acid, benzylpenicillin and ampicillin, 240 nm for carbenicillin, 260 nm for the other penicillins and 300 nm for imipenem.

When biphasic hydrolysis time courses were observed, initial and steady-state rates characteristic of the first and the second phases respectively (v_1 and v_2) and partial-inactivation pseudo-first-order rate constants (k_1) were determined with the help of the microcomputer-linked spectrophotometer as described by De Meester et al. (1987). The values of k_{cat} and K_m were obtained from single curves (De Meester et al., 1987) or by determination of the initial rates and analysis according to the Hanes equation. In some cases, K_m was so high that only the k_{cat}/K_m ratio could be determined. The re-activation of the OXA2 β -lactamase after the complete hydrolysis of cephaloridine or flomoxef was studied with oxacillin as a reporter substrate, and the different parameters (v_0 , v_1 and k_1) were determined as described by De Meester et al. (1987).

All experiments were performed at 30 °C in 50 mM sodium phosphate buffer, pH 7.0. Dilutions of the enzyme below a concentration of 0.1 mg/ml were performed with buffer solutions containing 0.1 mg/ml BSA.

RESULTS

Substrates obeying the Henri-Michaelis model

The hydrolysis of only three of the 22 studied antibiotics appeared to exhibit time courses corresponding to the integrated Henri-Michaelis equation. For these three compounds (oxacillin, ampicillin and cefaclor), the values of the kinetic parameters were not significantly different when determined on the basis of the analysis of either time courses or initial rates (Table 1), thus confirming a simple hyperbolic dependency of the rate upon the substrate concentration. In consequence, it could be safely assumed that, with these substrates, the classical hydrolysis pathway illustrated by Scheme 1 applied as it does with other active-site serine β -lactamases (Knott-Hunziker et al., 1982; Christensen et al., 1990) and they could thus be utilized as reporter substrates.

Substrates exhibiting complex hydrolysis time courses

Biphasic hydrolysis time courses were observed with many penicillins and cephalosporins, with flomoxef, a 7- α -methoxycephalosporin, and imipenem, a carbapenem. During the first phase the rate decreased faster than expected on the basis of the substrate depletion. This was followed by a 'normal' time course where the Henri-Michaelis model was obeyed. In all cases (but see below) and as shown before (Ledent et al., 1993), the burst was much larger than the enzyme concentration and, in consequence, could not be attributed to the accumulation of the acyl-enzyme, as sometimes observed with poor β -lactamase substrates

Table 1 Determination of the k_{cat} , K_m and k_{cat}/K_m parameters derived from initial-rate measurements (Hanes plot) or from analyses of complete hydrolysis time courses (integrated equation) for the three substrates obeying the Henri-Michaelis model

Substrate	Hanes plot			Integrated equation		
	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (μ M ⁻¹ ·s ⁻¹)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (μ M ⁻¹ ·s ⁻¹)
Oxacillin	900 $\pm$ 30	470 $\pm$ 20	1.9 $\pm$ 0.1	900 $\pm$ 80	360 $\pm$ 20	2.5 $\pm$ 0.2
Ampicillin	125 $\pm$ 14	60 $\pm$ 7	2.1 $\pm$ 0.2	110 $\pm$ 25	25 $\pm$ 8	4.4 $\pm$ 1.2
Cefaclor	88 $\pm$ 8	510 $\pm$ 50	0.17 $\pm$ 0.02	125 $\pm$ 13	750 $\pm$ 75	0.17 $\pm$ 0.02

Scheme 1 Interaction of β -lactamases with a 'normal' β -lactam antibiotic

E is the enzyme, S the β -lactam, ES the Henri-Michaelis complex, ES^* the acyl-enzyme and P the product of hydrolysis.

Figure 2 Hydrolysis time courses of 570 μ M cloxacillin (a), 750 μ M cephaloridine (c) and 150 μ M flomoxef (e)

Of the total amount of substrate, 35, 7 and 25% respectively were utilized at the end of the inactivation phenomenon. Each point represents the average of 12, 6 or 7 individual readings in (a), (c) and (e) respectively. Variations of $\ln v_t$ versus time are shown for cloxacillin (b) and cephaloridine (d). v_t is in $\Delta A \cdot s^{-1}$. To ensure that the patterns of residuals in parts (b) and (d) were random, we compared them with those described and attributed to rounding errors by Cardenas and Cornish-Bowden (1993). No similarities were found.

(Monnaie et al., 1992). Figure 2 presents examples of biphasic curves. The burst was particularly striking when the base-e logarithm of the instantaneous rate (v_t) was plotted against time. Figures 2(a) and 2(b) show the hydrolysis of cloxacillin where $\ln v_t$ decreased linearly after completion of the burst, thus indicating that, at that moment, the residual substrate concentration was smaller than $(K_m)_2$. Conversely, with cephaloridine (Figures 2c and 2d) and flomoxef (Figure 2e), a steady state was reached, implying $(K_m)_2$ values significantly lower than the residual substrate concentrations. Table 2 (which includes eqn. 1) summarizes experiments in which the values of the initial rates (v_1), those observed after completion of the burst (v_2) and the first-order rate constants (k_i) characteristic of the progressive inactivation were determined. When the burst was followed by a non-zero-order hydrolysis, an approximate 'steady-state' rate value was computed with the first data points following the completion of the burst.

Characteristic parameters of the second phase (Table 3)

These were calculated either as above by utilizing the first points after the completion of the burst and analysing the concentration-dependency of this rate with the help of the Hanes linearization of the Henri-Michaelis equation or, in the more favourable cases, by deducing the k_1 , $(k_{cat})_2$ and $(K_m)_2$ values from single curves. Figure 3 shows the analysis of one such hydrolysis curve obtained with carbenicillin. The k_1 value was deduced from the first part of this curve ($t < 50$ s) and the k_{cat} and K_m values from the second part ($t > 70$ s) of the same curve with the help of the integrated equation.

Effect of enzyme dilution

When concentrated enzyme (0.16 mg/ml) was added to 500 μ l of 50 mM sodium phosphate buffer, pH 7.0, containing 150 μ M cephaloridine to yield four different final enzyme concentrations ranging from 0.32 to 1.5 μ g/ml, no significant differences were found in the values either of the v_1/v_2 ratios (14.4 ± 0.9), nor of the partial inactivation rate constants, k_1 ($4.1 \pm 0.15 \times 10^{-2} s^{-1}$). Similar values of 13.3 ± 0.5 and $3.7 \pm 0.3 \times 10^{-2} s^{-1}$ respectively were found if the enzyme was first preincubated for 100 s at 30 $^{\circ}C$ at the final concentration of 1.5 μ g/ml before addition of concentrated cephaloridine to yield the same final substrate concentration.

Successive additions of substrate

Three substrates exhibiting biphasic kinetics (cephalosporin C, cephaloridine and benzylpenicillin) were hydrolysed and the v_1/v_2 ratios measured. After completion of the burst, a fresh sample of the same or of another substrate was added. When the first substrate was cephaloridine or cephalosporin C (high v_1/v_2 ratios), the second burst was much reduced when compared with the first one. In fact, when benzylpenicillin was the second substrate, even a small lag was observed ($v_1/v_2 < 1$). Conversely, when benzylpenicillin was the first substrate, the burst observed upon addition of one of the two cephalosporins remained large (Table 4).

Flomoxef was a substrate for which the value of the k_{cat}/K_m ratio corresponding to the first phase was 113 times greater than that of the second phase (Table 6 below). Flomoxef (20 μ M) was hydrolysed by the OXA2 β -lactamase (0.45 μ g). After completion of the burst, concentrated cephaloridine was added to 500 μ l of the mixture so that its initial concentration was 200 μ M. A lag was observed in the hydrolysis of cephaloridine (Figure 4), in

Table 2 Characteristics of the hydrolysis curves of substrates exhibiting biphasic kinetics

Abbreviations: 7-ACA, 7-Aminocephalosporanic acid; 6-APA, 6-Aminopenicillanic acid.

Substrate	[S] (μM)	$10^3 \times k_i$ (s^{-1})	v_1^*	v_2^*	v_1/v_2
Cephalosporins					
7-ACA	200	19	1.2	0.24	5.0
Cephaloglycin	100	19	5.6	0.9	6.2
Cephacetrile	170	31	62	10.4	6.0
Cephalosporin C	240	29	82	4.3	19
Cephalothin	200	5.8	13	6.7	1.9
Ceftazidime	100	41	0.21	0.035	6.0
Cefazolin	100	25	28	6.4	4.4
Cefamandole	170	4.5	15	10.2	1.5
Cefuroxime	140	5.2	0.09	0.013	7.0
Penicillins					
6-APA	2200	28	305	175	1.7
Benzylpenicillin	400	35	250	180	1.4
Carbenicillin	390	77	90	3.5	26
Methicillin	750	14	80	25	3.2
Cloxacillin	700	42	390	85	4.6
Dicloxacillin	1000	22	220	38	5.8

* v_1 and v_2 are expressed in $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. The different parameters were calculated with the help of eqn. (1): $(v_1 - v_2) = (v_1 - v_2) \times \exp(-k_i \times t)$; S.D. values did not exceed 10%.

Table 3 Kinetic parameters for the second phase

The different parameters were derived from the analysis of single curves (SCA) as described by De Meester et al. (1987) or by determining initial rates and utilizing the Hanes linearization of the Henri-Michaelis equation (H). S.D. values did not exceed 10%. 7-ACA and 6-APA are defined in Table 2.

Substrate	Concn. range (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	Method
7-ACA	50–300	0.27	260	1.0	H
Cephaloglycin	50–150	> 0.75	> 150	5.0	H
Cephalothin	20	3.40	7	485	SCA
Ceftazidime	5–100	0.02	12	1.7	H
Cefamandole	170	> 5.1	> 170	30	SCA
6-APA	2200	105	275	380	SCA
Benzylpenicillin	50	90	4	22500	SCA
Carbenicillin	390	2	150	13	SCA
Methicillin	100–750	15	200	75	H
Cloxacillin	650	> 45	> 650	69	SCA

contrast with the situation with a fresh sample of enzyme where a burst was observed with the same substrate.

Reversibility of the partial inactivation

The reversibility of the inactivation was demonstrated by the following experiments.

The enzyme (0.4 μg) was incubated with cephalosporin C, cephaloridine or benzylpenicillin until the burst was completed and was subsequently dialysed overnight at 4 °C against 50 mM sodium phosphate buffer, pH 7.0. Aliquots were then assayed against the same substrates. The reaction time courses did not significantly differ from those obtained with a protein solution similarly dialysed but without any prior contact with the substrate (Table 5).

Figure 3 Hydrolysis of 390 μM carbenicillin by 7.2 μg of β -lactamase (total volume = 0.5 ml)

(a) Hydrolysis time-course. (b) Plot of $\ln v_1$ versus time during the first 180 s showing that the inactivation phenomenon is completed after approx. 60 s. v_1 is in $\Delta A \cdot \text{s}^{-1}$. (c) Plot of $\ln(v_1 - v_2)$ versus time, allowing the calculation of k_i ($72.5 \times 10^{-3} \text{ s}^{-1}$); v_1 and v_2 were 87 and $3.6 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ respectively. (d) Plot of $(1/t) \cdot \ln \{ [S]_0 / ([S]_0 - [P]) \}$ versus $[P]/t$ constructed with the data recorded after completion of the inactivation phenomenon, yielding $(V_{\text{max}})_2$ ($5 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) and $(K_m)_2$ ($165 \mu\text{M}$). $[S]_0$ represents the initial concentration of substrate and $[P]$ the concentration of product at time t .

Table 4 v_1/v_2 ratios for successive reactions

Initial substrate concentrations were: cephalosporin C (300 μM), cephaloridine (150 μM) and benzylpenicillin (500 μM). The second addition was made after the burst was completed using a concentrated substrate solution so that it increased the concentrations of cephalosporin C, cephaloridine and benzylpenicillin by increments of 300, 150 or 500 μM respectively. Values are $\pm 10\%$.

First substrate	v_1/v_2 for the first substrate	v_1/v_2 for the second substrate		
		Cephalo- sporin C	Cephaloridine	Benzyl- penicillin
Cephalosporin C	11	1.4	3.7	0.9
Cephaloridine	15	1.7	2.3	0.7
Benzylpenicillin	1.4	9	12	0.8

Figure 4 Hydrolysis of 200 μM cephaloridine added to an enzyme/flomoxet mixture after completion of the burst

For details, see the text.

Table 5 v_1/v_2 ratios measured for different dialysed OXA2 solutions

The enzyme was incubated with the first substrate, the solution dialysed and assayed on the second substrate (see the text). Values are $\pm 10\%$.

Second substrate	First substrate ...	None	Cephalosporin C	Cephaloridine	Benzylpenicillin
Cephalosporin C (300 μ M)		8	7	8	8
Cephaloridine (150 μ M)		15	14	12	14
Benzylpenicillin (500 μ M)		1.2	1.2	1.4	1.2

Figure 5 Recovery of the initial properties of the enzyme after the complete hydrolysis of a cephaloridine solution

(a) Hydrolysis time course of 300 μ M cephaloridine by the OXA2 β -lactamase (0.8 μ g). The burst is not clearly visible under these conditions, since the progressive decrease of the reaction rate is due both to the inactivation phenomenon and the concomitant substrate depletion. (b) Value of the v_1/v_2 ratio when a sample taken in (a) was utilized to hydrolyse a fresh sample of cephaloridine.

The recovery of the initial properties of the enzyme after elimination of the substrate by completion of the hydrolysis reaction was also illustrated by the following experiment. The β -lactamase (0.8 μ g) was added to 400 μ l of 300 μ M cephaloridine and the mixture incubated at 30 $^{\circ}$ C. Aliquots of this solution (80 μ l) were sampled at different times and added to 400 μ l of the same substrate (200 μ M). As shown by Figure 5 and as already described above, the v_1/v_2 ratio in the second hydrolysis was much lower than in the first when the sample was taken before completion of the first reaction (450 s). After the complete disappearance of cephaloridine, this ratio continuously increased, indicating a progressive recovery of the initial properties of the enzyme.

As observed in an apparently similar case (but see below) by Citri et al. (1976), a pre-incubation with a 'burst' substrate induced the appearance of a lag in the hydrolysis of a 'normal' substrate (Figure 6a). Indeed, when oxacillin was added to an aliquot sampled from enzyme/cephaloridine or enzyme/flomoxef mixtures, a clear lag was observed in the hydrolysis of the second substrate, again indicating a progressive recovery of the initial

properties of the enzyme. A first-order rate constant characteristic of the re-activation phenomenon could be calculated (Figure 6b) with the help of eqn. (2) (note that eqn. 1 appears in Table 2):

$$(v_t - v_i) = (v_t - v_0) \times \exp(-k_r \times t) \quad (2)$$

where v_t was the rate of the reaction after complete recovery of the activity. k_r (re-activation rate constant) values of $4.4 (\pm 0.7) \times 10^{-3} \text{ s}^{-1}$ and of $0.86 (\pm 0.14) \times 10^{-3} \text{ s}^{-1}$ were found with cephaloridine and flomoxef as first substrates respectively.

Kinetic parameters for the initial and modified forms of the enzyme

The initial rates characteristic of the first (v_1) and second (v_2) phases were measured for substrate concentrations ranging from 10 to 150 μ M, and from 10 μ M to 2 mM, for cephaloridine and flomoxef, respectively. The values of the different constants calculated with the Hanes plot are given in Table 6.

By performing a complete hydrolysis of cephaloridine within 5 s with the help of the stopped-flow apparatus, the observed curves were little influenced by the inactivation phenomenon. On the basis of the integrated form of the Henri-Michaelis equation, the constants corresponding to the first phase could be directly determined: no significant differences were found in the k_{cat} ($37 \pm 5 \text{ s}^{-1}$) and K_m ($80 \pm 14 \text{ } \mu\text{M}$) values with four different initial substrate concentrations ranging from 30 to 105 μ M.

The hydrolysis of imipenem: presence of two bursts

The hydrolysis time-courses of imipenem were characterized by the presence of two subsequent bursts (Figure 7). For an imipenem concentration of 50 μ M, the first burst was completed after approx. 30 s, and the concentration of substrate then hydrolysed corresponded to $90 (\pm 20) \%$ of that of the enzyme. In consequence it could be attributed to the progressive accumulation of the acyl-enzyme. The second extended over a much larger period ($t_{1/2} = 170 \text{ s}$); the corresponding hydrolysed substrate concentration was larger than the enzyme concentration, indicating a branched pathway.

The hydrolysis of nitrocefin: a complex case

The hydrolysis time course of 400 μ M nitrocefin (Figure 8a) seemed to indicate a K_m value equal to, or larger than, the initial substrate concentration ($K_m \geq 400 \text{ } \mu\text{M}$). However, the initial rate remained independent of substrate concentrations ($v_0 = 74 \pm 6 \text{ } \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) between 4 and 160 μ M, indicating a K_m value lower than 4 μ M. Thus the time-dependent decrease of the rate of nitrocefin hydrolysis could not be attributed to substrate depletion. The possibility of a competitive product inhibition was studied by preparing a product solution with the help of the Zn^{2+} β -lactamase of *B. cereus* followed by addition of 100 μ M EDTA to inhibit completely the activity of this enzyme. Under these conditions, a linear relationship between v_0/v_i and $[P]$ was expected (eqn. 3):

$$\frac{v_0}{v_i} = 1 + \frac{K_m \times [P]}{(K_m + [S]_0) \times K_i} \quad (3)$$

where v_0 and v_i represent the rates of hydrolysis of the substrate (500 μ M oxacillin) in the absence or the presence of the hydrolysis product of nitrocefin $[P]$ and $[S]_0$ is initial substrate concentration.

The initial rates of hydrolysis of 500 μ M oxacillin were measured in the presence of product concentrations ranging from 19 to 83 μ M. The plot v_0/v_i versus $[P]$ was linear and allowed the determination of the affinity constant of the product

Figure 6 Hydrolysis of 1200 μM oxacillin by a sample of enzyme immediately after the hydrolysis of 10 μM cephaloridine

(a) Hydrolysis time course of oxacillin. (b) Plot of $\ln(v_t - v_t)$ versus time, yielding v_0 , v_t and k_t . The regression was performed from point 2 to point 9 and v_t determined between points 20 and 30. $k_t = 4.2 \times 10^{-3} \text{ s}^{-1}$; $v_0 = 130$; and $v_t = 420 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

Table 6 Kinetic parameters for cephaloridine and flomoxef

Parameter	Substrate ... Cephaloridine		Flomoxef	
	First phase	Second phase	First phase	Second phase
k_{cat} (s^{-1})	38 ± 4	1.2 ± 0.2	1.7 ± 0.2	0.05 ± 0.01
K_m (μM)	52 ± 8	67 ± 8	50 ± 5	170 ± 30
k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	730 ± 90	18 ± 2	34 ± 3.5	0.30 ± 0.05

for the enzyme ($K_i = 13 \pm 1 \mu\text{M}$), assuming a competitive inhibition. In the presence of a fixed product concentration, the inhibition decreased when the substrate concentration increased, indicating a competition between oxacillin and the hydrolysis product of nitrocefin; for example, for a product-concentration ($[P]$) value of 100 μM , v_i corresponded to 22 % and to 43 % of v_0 at oxacillin concentrations of 500 and 2000 μM respectively. These two results clearly indicated the presence of a competitive product inhibition. However, another phenomenon was also present, since an additional inhibition (Table 7) was observed when a concentrated solution of fresh substrate was added to an OXA2 sample just after completion of the hydrolysis of 20 μM nitrocefin. Moreover, in the case of a strict competitive product inhibition, a linear relationship between $([S]_0 - [S])/t$ and $(1/t) \times \ln([S]_0/[S])$ must be found (eqn. 4; Orsi and Tipton, 1980):

$$\frac{[S]_0 - [S]}{t} = \frac{V_{\text{max.}}}{1 - (K_m/K_i)} - K_m \times \frac{K_i + [S]_0}{K_i - K_m} \times \frac{1}{t} \times \ln\left(\frac{[S]_0}{[S]}\right) \quad (4)$$

Figure 8(b) shows that this was not the case for the hydrolysis of nitrocefin. Indeed, a burst similar to those observed with other substrates was clearly visible under conditions where the amount

Figure 7 Hydrolysis of 50 μM imipenem by 1.9 μM OXA2 β -lactamase

(a) Rapid burst ($\Delta[S] = 1.6 \mu\text{M}$) during the first 100 s of the reaction. (b) Second burst recorded on a longer time scale showing the progressive rate decrease ($\Delta[S] = 13 \mu\text{M}$). $\Delta(S)$ represents the concentration of substrate consumed at the end of each burst determined by extrapolating the apparent steady state to zero time.

Figure 8 Hydrolysis of 400 μM nitrocefin

(a) Hydrolysis time course. (b) Plot of $(1/t) \times \ln([S]_0/[S])$ versus $[P]/t$.

of product formed during the partial inactivation was very low (Figure 9).

DISCUSSION

The reaction catalysed by the OXA2 β -lactamase appeared to obey the integrated form of the Henri-Michaelis equation with only three of the 22 studied substrates. 'Burst' kinetics were indeed observed with most β -lactams. Penicillins were generally

Table 7 Influence of the hydrolysis product of nitrocefin on the rate of hydrolysis of various substrates

The relative initial hydrolysis rates of cephaloridine (100 μ M), nitrocefin (100 μ M), benzylpenicillin (100 μ M) and oxacillin (500 μ M) were measured in absence of product and in presence of 20 μ M product. They were also recorded when a concentrated solution of fresh substrate was added to an OXA2 sample just after completion of the hydrolysis of 20 μ M nitrocefin (20*). ND, not determined.

Substrate	Substrate concn. (μ M) ...	Relative initial hydrolysis rate		
		0	20	20*
Cephaloridine	100	100	69 $\pm$ 9	ND
Nitrocefin	100	100	87 $\pm$ 6	35 $\pm$ 7
Benzylpenicillin	100	100	82 $\pm$ 3	59 $\pm$ 2
Oxacillin	100	100	67 $\pm$ 7	ND

Figure 9 Hydrolysis of 80 μ M nitrocefin

After 90 s, the concentration of product was less than 2 μ M. A k_1 value of $61 \times 10^{-3} \text{ s}^{-1}$ was found.

more sensitive to hydrolysis than cephalosporins. Nonetheless, the initial rates (v_1) determined for cephalosporin C, cephacetrile and cephaloridine were similar to those observed for carbenicillin and methicillin; the v_2 value for carbenicillin was in the range of those obtained for cephalosporins; the k_{cat}/K_m ratio corresponding to the second phase for cephalothin was much larger than those for a series of penicillins. If one considers this latter constant, benzylpenicillin and, to a lesser extent, ampicillin, were better substrates than oxacillin.

The k_{cat} value for ampicillin and those corresponding to the second phase for benzylpenicillin and 6-aminopenicillanic acid were very similar. But the removal of the acyl group on the C-6 position, yielding 6-aminopenicillanic acid, significantly increased the K_m value. Replacing the NH_2 group of the side chain of ampicillin by a carboxylic moiety both decreased k_{cat} and increased K_m and also induced the appearance of a burst.

With the exception of the first 'burst' observed with imipenem, the concentration of substrate hydrolysed during the inactivation phenomenon was always larger than the enzyme concentration, indicating a branched pathway. As in the experiments described by Citri *et al.* (1976), the partial inactivation of the enzyme persisted for some time after completion of the hydrolysis reaction, but was eventually fully reversible. The fact that the rate of re-activation of OXA2 was slow compared with the turnover recorded during the second phase implied that the

Scheme 2 Model explaining biphasic hydrolysis with a branched pathway at the level of the acyl-enzyme, with the condition: $k_3 \gg k_{+4} > k_{-4}$

This model is characterized by the following equations:

$$(k_{\text{cat}})_1 = \frac{k_2 \cdot k_3}{k_2 + k_3} \quad (5)$$

$$(K_m)_1 = \frac{k_3 \cdot K'}{k_2 + k_3} \quad (6)$$

$$(k_{\text{cat}})_2 = \frac{k_2 \cdot k_3}{k_2 \cdot (1 + K_1) + k_3} \quad (7)$$

$$(K_m)_2 = \frac{k_3 \cdot K'}{k_2 \cdot (1 + K_1) + k_3} \quad (8)$$

$$K_1 = \frac{k_{+4}}{k_{-4}} \quad (9)$$

$$K' = \frac{k_{-1} + k_2}{k_{+1}} \quad (10)$$

recovery of the initial activity did not occur by a simple deacylation of a rather stable acyl-enzyme, as illustrated by Scheme 1. Different possibilities of branched pathways were then tested.

For mechanisms with an equilibrium between monomeric and dimeric forms of free enzyme or between two different states of a free monomer (Scheme 3), preincubation of the enzyme alone should eliminate the 'burst' (Waley, 1991). This was not the case for the hydrolysis of cephaloridine by preincubated OXA2. Moreover, for a monomer-dimer equilibrium, the inactivation rate constant, k_i , should depend on the enzyme concentration (Neet and Ainslie, 1980). This result confirms that of Ledent *et al.* (1993), who showed that, despite its abnormally apparent high molecular mass determined by gel filtration, OXA2 did not behave as a dimer in rapid equilibrium with a monomer (Dale and Smith, 1976).

The model generally accepted to explain biphasic hydrolysis by β -lactamases implies either a partial unfolding of the acyl-enzyme or a progressive accumulation of a stable acyl-enzyme after the departure of the 3'-leaving group of cephalosporins (Scheme 2). With such a model, the k_{cat} and K_m values recorded at the steady state are smaller than those observed under initial-rate conditions, but the k_{cat}/K_m ratio remains constant (see eqns. 5–8 and Scheme 2). The results obtained for OXA2 with cephaloridine and flomoxef showed that the decrease of the k_{cat} values between the first and the second phases was not accompanied by a parallel decrease of K_m , which made this mechanism inadequate for explaining the behaviour of the OXA2 β -lactamase.

The large range of the v_1/v_2 ratio values observed for the 'burst' substrates (from 1.4 for benzylpenicillin to about 100 for flomoxef) could be due to: (i) a transition of the enzyme to a unique modified conformation exhibiting modified catalytic properties; (ii) a transition to other conformations, each specific

Scheme 3 Reaction pathway with a branch at the level of the free enzyme

Z represents either another conformer, or a dimer, of E.

to each particular substrate; or (iii) a more or less important displacement of an equilibrium between two enzyme forms, characterized by different efficiencies. The results obtained upon successive additions of substrates rule out the first possibility, since in this case no 'burst' would have been observed after addition of cephaloridine or cephalosporin C to the enzyme after partial inactivation by benzylpenicillin and no 'lag' after addition of cephaloridine to the flomoxef-inactivated enzyme. Distinction between the two other possibilities cannot be made on the basis of the present kinetic analysis.

The behaviours of two of the 'burst' substrates were even more complex. The hydrolysis of imipenem was characterized by the occurrence of two subsequent 'bursts'. The first one corresponded to a progressive acyl-enzyme accumulation that was slow enough to be recorded with the help of a standard spectrophotometer. The second one was larger than the enzyme concentration and indicated a branched pathway. The very different K_m values observed upon monitoring of the hydrolysis of nitrocefin under initial-rate conditions or by analysing complete time courses were due to a partial substrate-induced inactivation complicated by a competitive inhibition by the hydrolysis product.

In the present study, the catalytic properties of the OXA2 β -lactamase were studied with a large number of substrates. 'Burst' kinetics prevailed with many penicillins, cephalosporins and with flomoxef and imipenem. The model generally accepted to explain such a behaviour and implying the existence of a branch at the level of the acyl-enzyme failed to account for the results obtained with the OXA2 β -lactamase. Mechanisms involving an equilibrium between two different forms of the free enzyme could also be ruled out. A more complex model probably prevails for this enzyme, and is presently under detailed investigation.

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