The kinetics of non-stoichiometric bursts of β-lactam hydrolysis catalysed by class C β-lactamases

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Class C β-lactamases from *Pseudomonas aeruginosa* and several species of the Enterobacteriaceae have been observed to undergo a rapid burst in hydrolysis of β-lactam antibiotics before relaxation to a steady-state rate of hydrolysis. The amplitude of the burst corresponds to the hydrolysis of between 1 and 10000 mol of the substrate per mol of enzyme. The decay of the rate of hydrolysis in the burst phase comprises two exponential reactions, which indicates that there are three different reactive states of the enzymes. Examination of the kinetics of acylation by slowly reacting β-lactams suggests that there are three forms of the free enzyme in slow equilibrium. Thus it would appear that the burst kinetics exhibited by class C enzymes can be attributed to redistribution of the enzyme between different conformations induced by the reaction with substrate.

INTRODUCTION

One of the principal causes of resistance of Enterobacteriaceae and Pseudomonaceae to β-lactam antibiotics has proved to be the expression of high levels of enzymes that actively degrade the compounds. The β-lactamases produced by these organisms are frequently chromosomally encoded enzymes belonging to class C as described by Ambler (1980). The affinities of the different enzymes and their different kinetic properties have been extensively discussed in recent years (Coulson, 1985; Joris et al., 1988; Galleni et al., 1988; Bush, 1989a,b,c; Matagne et al., 1990). The crystal structure of one enzyme representative of this class has been determined (Oefner et al., 1990) and site-directed mutagenesis aimed at understanding the roles of particular residues has been undertaken (Tsukamoto et al., 1990a,b). An integral part of the description of the mechanism is identification of the intermediates of the catalytic cycle and determining their rates of interconversion. Here, analysis of the steady-state and transient kinetics of parent and mutant proteins can provide much useful information (Cartwright et al., 1989; Christensen et al., 1990; Virden et al., 1990). A problem that frequently has been encountered in the analysis of kinetics of β-lactamases has been the observation of non-stoichiometric bursts of hydrolysis, that is to say, bursts of hydrolysis representing multiple turnovers of the enzyme (Citri et al., 1976; Charnas and Then, 1988; Galleni et al., 1988; Matagne et al., 1990). The burst in hydrolysis usually has been attributed to an isomerization of the acyl-enzyme complex that leads to a branching of the pathway (Kiener et al., 1980; Carrey et al., 1984; Faraci and Pratt, 1985; Persaud et al., 1986; Fink et al., 1987; Charnas and Then, 1988; Matagne et al., 1990; Monnaie et al., 1992), although the possibility that it also involves an isomerization of the free form of the enzyme has been raised (Persaud et al., 1986). Thus there are three possibilities: (i) isomerization of the free form of the enzyme between catalytically competent conformations; (ii) isomerization of the acyl enzyme; and (iii) isomerization of both free and bound species. The first two can be readily distinguished by simple kinetic tests (Waley, 1991). Distinguishing between the latter two should be feasible in that only one form of enzyme should be present before addition of substrate if only the acyl-enzyme can isomerize. Distinguishing between the first and last models is not possible by simple kinetic tests, as two forms of the enzyme will always be present. It now appears that class C β-lactamases do indeed exist as equilibrium mixtures of conformations that have different reactivity towards, and affinity for, β-lactam antibiotics. Thus either the free form of the enzyme alone is able to isomerize or the enzyme can isomerize as both the free form and as the acyl-enzyme intermediate.

EXPERIMENTAL

Bacterial strains

Escherichia coli SN01 and *E. coli* SN03 pAD7 were obtained from S. Normark (University of Washington, St. Louis, MO, U.S.A.) and express AmpC β-lactamase (Normark and Burman, 1977; Jaurin et al., 1981), *Citrobacter freundii* 1203 was obtained from B. Wiedemann (University of Bonn, Bonn, Germany), *E. cloacae* 908R and *E. cloacae* M6300 are respectively laboratory and clinical isolates selected by exposure to β-lactam antibiotics (Then and Angehrn, 1986; Follath et al., 1987); the differences between β-lactamases expressed by these organisms have been described (Then et al., 1988). *Morganella morganii* V1627 and *Providencia rettgeri* 5298 were obtained from W. Cullmann (of this Company), *Pseudomonas aeruginosa* 18SH was obtained from W. Zimmermann (Ciba–Geigy Ltd., Basel, Switzerland), *P. aeruginosa* 143724 and *P. aeruginosa* 143811R were obtained from P. Angehrn (this Company).

Methods

Purification

The standard purification procedure was adapted from the method described by Cartwright and Waley (1984), as follows. Frozen cell paste was thawed and resuspended at 10–20 g/100 ml in 20 mM Tris/10 mM $MgSO_4$ adjusted to pH 7.4 with 4 M HCl containing 10 μg/ml bovine pancreatic DNAase. The cells were broken by a single passage through a French pressure cell at

Abbreviations used: CENTA, 7-(thienyl-2-acetamido)-3-(3-carboxy-4-nitrophenylthio)methyl-3-cephem-4-carboxylic acid; FAP, 6β-furylacryloylamidopenicillanic acid.

about 2000 MPa and 4 °C. Membranes and cell debris were removed by centrifugation at 110000 *g* for 90 min, and the supernatant was dialysed against three changes of 10 mM Tris/HCl, pH 7.0. The sample was then applied to a column of S-Sepharose Fast Flow equilibrated with the same buffer. The β-lactamase activity was eluted with a gradient of 10–100 mM Tris/HCl, with a single peak usually occurring at approx. 75 mM Tris. The fractions containing the activity were applied directly to a column of 4-aminophenylboronate coupled to Affigel 10 from Bio-Rad equilibrated with 20 mM triethanolamine adjusted to pH 7.0 with 4 M HCl and eluted with a gradient of 0–0.5 M sodium borate in the same buffer. The purified enzyme was stored at 4 °C in 0.1 M sodium phosphate buffer, pH 7.0, at a concentration of between 0.5 and 5 mg/ml.

Analytical ultracentrifugation

The determination of sedimentation coefficients and molecular masses of the various β-lactamases was performed by Mr. Ariel Lustig at the Biocentre of Basel University.

Kinetic measurements

All kinetic measurements were performed at 37 °C in 0.1 M sodium phosphate (0.061 M Na_2HPO_4/0.039 M NaH_2PO_4, pH 7.0). The rates of hydrolysis were determined spectrophotometrically with either a Kontron Uvikon 860 spectrophotometer or a Perkin–Elmer λ2 spectrophotometer coupled to an Epson PC2Xe personal computer for data capture and analysis. Kinetic parameters were estimated from time courses recorded from reaction mixtures containing different initial concentrations of the substrate using direct weighted fits to the Michaelis–Menten equation (Leatherbarrow, 1989). In some cases the initial rate of hydrolysis was monitored spectrophotometrically in reaction mixtures that had been created used an Applied Photophysics stop-flow accessory. This enabled reactions to be monitored over the range 0.5–2 s. In other cases, the approach to the steady-state rate of hydrolysis was monitored over the range 5–1000 ms, using a Hi-Tech (Salisbury, U.K.) MF3 multi-mixing stopped-flow spectrofluorimeter coupled to an Epson PC2Xe personal computer for data capture and analysis. The following wavelengths and absorption coefficients were used (Charnas and Then, 1988; Matagne et al., 1990; Virden et al., 1990): benzylpenicillin (purchased from Sigma Chemical Co., Buchs, Switzerland) $\Delta\epsilon_{237}$ $-560\ M^{-1}\cdot cm^{-1}$; cefbuperazone, $\Delta\epsilon_{265}$ $-5500\ M^{-1}\cdot cm^{-1}$; ceftriaxone (F. Hoffmann–La Roche), $\Delta\epsilon_{255}$ $-7700\ M^{-1}\cdot cm^{-1}$; 7-(thienyl-2-acetamido)-3-(3-carboxy-4-nitrophenylthio)methyl-3-cephem-4-carboxylic acid (CENTA; purchased from Calbiochem, Lucerne, Switzerland), $\Delta\epsilon_{415}$ $9900\ M^{-1}\cdot cm^{-1}$; cephalothin (Sigma), $\Delta\epsilon_{265}$ $-6700\ M^{-1}\cdot cm^{-1}$; 6-β-furylacryloylamidopenicillanic acid (FAP; purchased from Calbiochem), $\Delta\epsilon_{340}$ $-1730\ M^{-1}\cdot cm^{-1}$; nitrocefin (Becton Dickinson and Co.), $\Delta\epsilon_{482}$ $17400\ ^{-1}\cdot cm^{-1}$.

The rate of acylation of the enzyme by slow-reacting substrates was determined by monitoring the decrease in residual activity towards nitrocefin, as follows. A 5 ml sample of 0.5 mM enzyme solution in 0.1 M sodium phosphate buffer containing 0.01 mg/ml BSA was rapidly mixed with 5 ml of a solution of the substrate in the same buffer and then incubated at 37 °C for an interval of between 1 and 60 s. The reaction was stopped by the rapid addition of 1 ml of 100 μM nitrocefin solution in the assay buffer, and the mixture was rapidly transferred to the spectrophotometer, where residual activity towards nitrocefin was assayed for 1 min.

RESULTS

General characteristics of the burst in hydrolysis

Several class C β-lactamases showed clearly biphasic time courses of reaction with the rapidly hydrolysed penicillin FAP (Figure 1) and with some cephalosporins, for example cefbuperazone (Figure 2). The enzymes exhibited a less pronounced, but nevertheless reproducibly detectable, decay of the initial rate to a slower steady-state rate in reactions with other penicillins and cephalosporins (results not shown). The relative amplitudes of the burst shown by the enzymes with different substrates were rather similar: for example, the enzyme from *E. cloacae* 908R showed the largest amplitude with all substrates (Table 1). Non-stoichiometric bursts (that is, having an amplitude greater than the amount of enzyme) can arise when the enzyme is converted into a less active form after repeated turnovers of the catalytic cycle

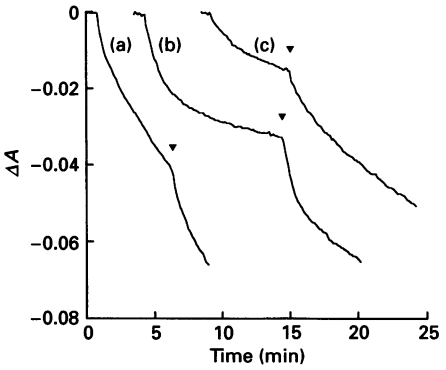


Figure 1 Time courses of FAP hydrolysis

(**a**) *P. rettgeri* (10 nM), (**b**) *E. cloacae* 908R (5 nM), (**c**) *M. morganii* (1 nM). The FAP concentration was 10 μM in each experiment. The arrowheads (▼) indicate the addition of a second aliquot of enzyme.

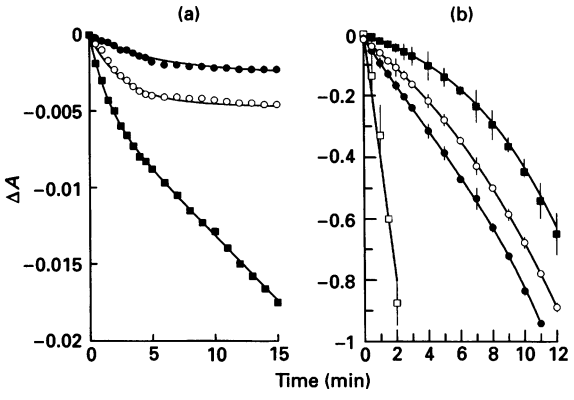


Figure 2 Time course of cefbuperazone hydrolysis by *E. coli* SN03 β-lactamase

(**a**) Direct observation of cefbuperazone hydrolysis; the curves were calculated from kinetic parameters (Tables 2 and 3) obtained by direct weighted fit to eqn. (1). The cefbuperazone concentrations were 10 (●), 20 (○) and 40 (■) μM. (**b**) Hydrolysis of 10 μM cephalothin in the presence of cefbuperazone. The reaction was started by adding enzyme simultaneously to the preformed mixture of substrates and to a reference cuvette containing the appropriate concentration of cefbuperazone. The curves were calculated according to eqn. (5), using the kinetic parameters given in Tables 2 and 3. The cefbuperazone concentrations were 0 (□), 10 (●), 20 (○) and 40 (■) μM; the points are the means of three determinations.

Table 1 Amplitudes of the burst observed with class C β-lactamases

The maximum amplitude, determined by extrapolation from a series of concentrations, is given in mol of substrate hydrolysed/mol of enzyme. ND indicates that no burst was detected under the conditions tested, and a minus sign indicates that this combination was not tested.

Enzyme source	Substrate ...	Amplitude (molar equiv.) FAP	Benzylpenicillin	Ceftriaxone	Cefbuperazone
E. coli SN01		5330 ± 100	15 ± 3	ND	23 ± 5
E. cloacae 908R		6500 ± 400	2136 ± 256	2.5 ± 0.9	35 ± 4
E. cloacae M6300		5700 ± 300	1895 ± 174	1.9 ± 0.5	30 ± 6
C. freundii		3700 ± 230	1200 ± 98	1.85 ± 0.33	26 ± 2
M. morganii		6300 ± 297	–	ND	12 ± 3
P. rettgeri		250 ± 15	–	ND	2.8 ± 0.5
P. aeruginosa 18SH		3.52 ± 0.71	ND	ND	ND
P. aeruginosa 811R		1194 ± 278	–	1.1 ± 0.3	

Table 2 Steady-state kinetic parameters

Enzyme	Reaction in initial phase $k_{cat.}$ (s^{-1})	K (μM)	$k_{cat.}/K$ ($s^{-1} \cdot \mu M^{-1}$)	Reaction in continuing phase $k_{cat.}$ (s^{-1})	K (μM)	$k_{cat.}/K$ ($s^{-1} \cdot \mu M^{-1}$)
FAP						
E. coli SN01	222 ± 27	220 ± 29	0.67 ± 0.08	110 ± 21	800 ± 41	0.14 ± 0.02
E. cloacae 908R	433 ± 36	280 ± 17	1.54 ± 0.10	53 ± 8	650 ± 28	0.08 ± 0.01
C. freundii	870 ± 56	740 ± 23	1.18 ± 0.06	24 ± 2	210 ± 34	0.11 ± 0.02
Ceftriaxone						
E. coli SN01	0.30 ± 0.05	0.10 ± 0.03	3.0 ± 0.9	(no second phase)		
E. cloacae 908R	0.18 ± 0.07	0.10 ± 0.01	1.8 ± 0.9	0.063 ± 0.011	0.20 ± 0.03	0.30 ± 0.03
C. freundii	0.15 ± 0.02	0.08 ± 0.01	1.9 ± 0.1	0.038 ± 0.02	0.17 ± 0.02	0.24 ± 0.01
Cefbuperazone						
E. coli SN01	0.29 ± 0.02	0.11 ± 0.02	3.0 ± 0.1	0.16 ± 0.03	200 ± 81	0.0008 ± 0.0001
[Cefbuperazone in the presence of cephalothin]		[0.32 ± 0.05]			[300 ± 38]	
Nitrocefin						
E. coli SN01	639 ± 58	360 ± 72	1.78 ± 0.15	39 ± 8	2.4 ± 0.3	15.7 ± 1.8
CENTA						
E. coli SN03	1312 ± 159	833 ± 66	1.55 ± 0.25	293 ± 68	49 ± 12	5.98 ± 4.2
Cephalothin						
E. coli SN03	757 ± 88	380 ± 51	1.99 ± 0.17	23 ± 4	9.8 ± 1.1	2.4 ± 0.3
Cephalothin in the presence of cefbuperazone						
E. coli SN01	560 ± 59	370 ± 62	1.51 ± 0.22	70 ± 0.12	25 ± 6	2.8 ± 0.7

* The values are inhibition constants (K_I and K_{II} from eqn. 5)

† The values entered are for the high- and low-affinity branches of the biphasic Eadie–Hofstee plots (see text and Figure 3).

or because of inhibition through accumulation of product (Fersht, 1985).

The rapid burst of FAP hydrolysis was followed by a long slower phase of reaction that continued until a significant fraction of the substrate had been consumed. The addition of a second aliquot of protein during the latter phase resulted in a repetition of the burst (Figure 1). The amplitude of the burst was proportional to enzyme concentration in the range where it was practical to detect it (results not shown). These observations suggested that the slower reaction was not due to the effect of either accumulated product or depleted substrate, that the burst was not due to a single rapid turnover followed by rate-limiting release of hydrolysed product and that protein–protein interactions do not influence this behaviour. Two mechanisms which lead to accumulation of an inactive form of the enzyme have been suggested for β-lactamases (reviewed by Waley, 1991). These may be summarized as (1) branching of the reaction pathway by isomerization of the acyl-intermediate and (2) operation of parallel reaction pathways through isomerization of the free form of the enzyme.

Steady-state kinetic parameters in the different phases of the reaction

The kinetic parameters describing the rate of reaction in the two phases are expected to be different in reactions branching at the acyl intermediate. The magnitude of $k_{cat.}/K_m$ is predicted to be smaller in the initial phase than it is in the steady-state phase

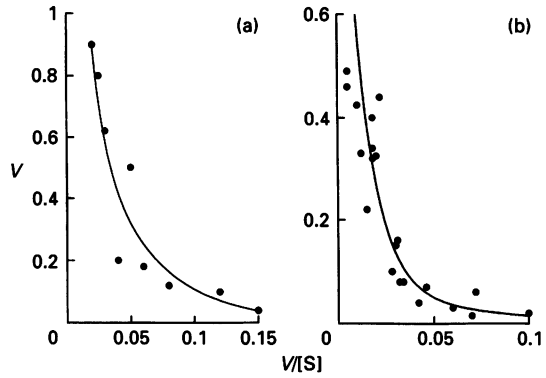


Figure 3 Eadie–Hofstee plots of substrate dependence of the reaction between *E. coli* SN03 β-lactamase and (a) nitrocefin or (b) CENTA

The velocity of reaction (V, as μmol of substrate/s per nmol enzyme) is plotted as a function of V/[substrate concentration] (V/[S] as l/s per nmol of enzyme). The curves were calculated according to eqn. (3), using the kinetic parameters given in Table 2.

(Waley, 1991). This is not necessarily true for reactions involving isomerization of the free form of the enzyme.

The kinetic parameters were determined for the two phases by fitting reaction time courses obtained at different substrate concentrations ([L]) to eqn. (1) (Neet and Ainslie, 1980):

$$V_t = \frac{V_1[L]\cdot e^{-\alpha t}}{K_1+[L]} + \frac{V_2[L]\cdot(1-e^{-\alpha t})}{K_2+[L]} \quad (1)$$

which is a general equation describing an exponential decay (with time constant α) from one steady-state rate of hydrolysis (V_1, K_1) to another (V_2, K_2). V_1 and V_2 are maximum velocities, dependent on the rate constants for catalysis and enzyme concentration. The steady-state kinetic parameters are potentially composite parameters depending on the abundance of different forms of the enzyme at the beginning of the reaction and after the final steady-state has been established (Ricard et al., 1974; Neet and Ainslie, 1980). The time constant, α, is a composite of the rate of interconversion between the two active states, the rate constants for catalysis and substrate concentrations.

The instantaneous velocity, v_t, was estimated by fitting intervals of the absorption trace to the straight line:

$$[P_t] = v_t\cdot t + [P_0] \quad (2)$$

where $[P_0]$ is the product concentration at the onset of the interval. The intervals were between 0.1 and 10 s, according to the velocity of the reaction.

With most substrates, the rapid phase of hydrolysis had not only a higher $k_{cat.}$ than did the slower phase, but it also had a different K_m (Table 2). In some instances the K_m was lower for the fast phase of the reaction, and therefore the values of $k_{cat.}/K_m$ were either higher for the initial phase, or not significantly different in the two phases. Even for those substrates where the time course of hydrolysis was monophasic, non-linearities in the dependence of rate of reaction on substrate concentration were frequently apparent. Thus substrates like nitrocefin and CENTA, which are rapidly and apparently monophasically hydrolysed, showed strongly curved plots of substrate-dependence (Figure 3 and Table 2). Such a dependence is readily explained by the presence of two or more forms of the free enzyme with different kinetic properties. The substrate-dependence of the rate of hydrolysis by such a system is given by eqn. (3):

$$V_{obs.} = \frac{V_1[L]}{K_1+[L]} + \frac{V_2[L]}{K_2+[L]} \quad (3)$$

Burst rate constant

Waley (1991) suggested that the time constant characterizing the transient burst (α) can be used to distinguish between simple models for branched pathways. The time constant (α) is expected to increase with substrate concentration if the mechanism branches at the acyl-enzyme intermediate, but to decrease with substrate concentration if substrate promotes isomerization to an alternative free form of the enzyme. The substrate-dependence is more difficult to predict for a reaction mechanism with multiple active free forms of the enzyme, as the behaviour depends very much on the relative rates of reaction of the different forms of the enzyme (Ricard et al., 1974). Simulation, using the methods described by Waley (1991) has shown that there may be little or no dependence of α on substrate concentration with many combinations of rate constants.

The observed value of α decreased slightly with substrate concentration for the reaction with penicillins and was independent of substrate concentration with the cephalosporins that were tested (Table 3).

In the reaction between *C. freundii* β-lactamase and FAP (Figure 4), the burst was clearly biphasic and had to be fitted to a modified form of eqn. (1), which assumes a double-exponential relaxation to the final linear rate of hydrolysis (eqn. 4).

$$[v_t] = v_1\cdot e^{-\gamma t} + [v_2\cdot e^{-\beta t} + v_3\cdot(1-e^{-\beta t})](1-e^{-\gamma t}) \quad (4)$$

Table 3 Concentration-dependence of the burst rate (k_b)

The time constant describing the burst was determined by weighted fits to eqn. (1).

		α (min^{-1})								
Reaction (substrate + enzyme)	s (μM)...	5	10	20	40	50	60	100	200	500
FAP + *E. coli* SN03		–	–	2.82 ± 0.18	2.10 ± 0.31	–	2.10 ± 0.23	2.10 ± 0.25	–	1.92 ± 0.11
Benzylpenicillin + *E. coli* SN03		18.0 ± 2.1	–	–	–	13.8 ± 1.8	–	13.8 ± 1.9	13.8 ± 2.2	–
Cefbuperazone + *E. coli* SN03		0.40 ± 0.05	0.59 ± 0.06	0.67 ± 0.05	0.72 ± 0.15	–	–	–	–	–
Cefbuperazone + 100 μM cephalothin + *E. coli* SN03		0.28 ± 0.03	0.19 ± 0.02	0.30 ± 0.08	0.30 ± 0.05	–	–	–	–	–
Ceftriaxone + *E. cloacae* 908R		0.98 ± 0.11	1.11 ± 0.12	0.89 ± 0.08	1.02 ± 0.09	–	–	–	–	–

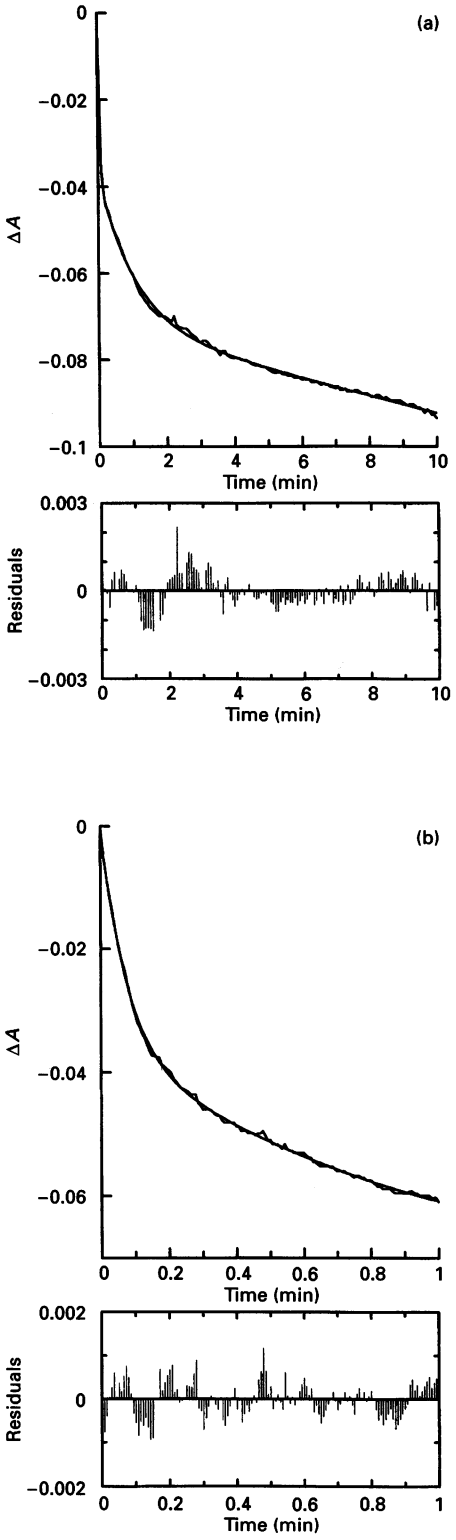
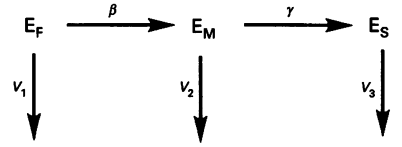


Figure 4 Time course of hydrolysis of FAP by *C. freundii* 1203 β-lactamase

Tracings of the absorption change during (**a**) the approach to the steady-state rate of hydrolysis and (**b**) the initial transient of the burst in hydrolysis are shown on separate time scales. In both cases the reaction was started by rapidly mixing equal volumes of 100 μM FAP and 10 nM β-lactamase, both dissolved in sodium phosphate buffer. The smooth curve in (**a**) was calculated from parameters derived by fitting the data to eqn. (4). The smooth curve in (**b**) was calculated from parameters obtained by fitting the absorption data to a double-exponential decay.



Scheme 1 Reaction scheme for an enzyme existing in three catalytically competent conformations (denoted E_F, E_M and E_S)

Each form can catalyse β-lactam hydrolysis with its own set of kinetic parameters (reaction velocities V_1, V_2 and V_3 are indicated). The forms interconvert with time constants β and γ, which are comprised of combinations of rate constants describing the protein isomerization reactions, the rate constants describing catalysis of β-lactam hydrolysis and substrate concentrations (Neet and Ainslie, 1980; Waley, 1991).

v_1, v_2 and v_3 represent the steady-state rates of turnover in each phase of the reaction, and time constants β and γ describe the rate of conversion of one reactive state to the next as shown in Scheme 1.

The reaction of the *E. cloacae* 908R and *P. rettgeri* enzymes with benzylpenicillin could also be fitted to this model, but the difference between this and a single-exponential model was not significant (Table 4). Such progress curves can arise from the presence of three different reactive states of the enzymes which interconvert slowly during the course of the reaction.

Availability of the free form of the enzyme during the slow reaction

One of the principal differences between the mechanisms under discussion lies in the intrinsic reactivity of the free form of the enzyme. In the mechanism where the pathway branches at the acyl-enzyme, the low rate of hydrolysis is principally due to accumulation of a significant fraction of the enzyme as an unreactive acyl intermediate. There is no change in intrinsic reactivity of the free form of the enzyme, although its concentration tends towards zero. In contrast, the mechanism involving the isomerization of the free form allows the enzyme to accumulate as forms that have different intrinsic reactivities. In the former mechanism, the free form of the enzyme returns only after most of the inhibiting substrate has been hydrolysed. In the latter mechanism, all of the enzyme can pass through a free form during turnover, although its intrinsic reactivity and abundance may be different at different times in the reaction.

Burst kinetics were observed in the reaction between several class C enzymes and cefbuperazone (Figure 2). When the reaction of the same concentration of cefbuperazone was monitored in the presence of cephalothin, the initial rate of cephalothin hydrolysis was low, because of competition from cefbuperazone but, as reaction proceeded, the rate accelerated with the same rate of change as the decrease in activity towards cefbuperazone occurred. The reaction can be described by modification of eqn. (1) to allow for competitive inhibition from cefbuperazone (eqn. 5).

$$V_t = \frac{V_1[\mathrm{L}]\cdot(e^{-\alpha t})}{K_1(1+[\mathrm{I}]/K_\mathrm{I})+[\mathrm{L}]} + \frac{V_2[\mathrm{L}]\cdot(1-e^{-\alpha t})}{K_2(1+[\mathrm{I}]/K_\mathrm{II})+[\mathrm{L}]} \qquad (5)$$

The values of V_1, K_1 and V_2, K_2 describe cephalothin hydrolysis by the two populations of enzyme, and they should be compared with the parameters obtained for the two phases of substrate dependence observed in Eadie–Hofstee plots (Table 2). The values of K_I and K_II describe the interaction with cefbuperazone during the two phases of reaction and should be compared with

Table 4 Biphasic burst kinetics

The time courses of hydrolysis were recorded at a single concentration of substrate five times the K_m of the steady-state phase of hydrolysis. The curves were fitted either with a single exponential decay to the steady-state rate (eqn. 1) or to a double-exponential decay (eqn. 4). χ^2 indicates the value of reduced χ^2 generated by the fitting.

	Model eqn. (4)			Model eqn. (1)	
Reaction	γ (min^{-1})	β (min^{-1})	χ^2	α (min^{-1})	χ^2
FAP + *E. coli* SN01	ND*			1.83 ± 0.03	4×10^{-8}
FAP + *E. cloacae* 908R	1.44 ± 0.44	0.598 ± 0.060	5×10^{-8}	0.768 ± 0.014	2×10^{-7}
FAP + *C. freundii*	200 ± 58	0.859 ± 0.075	6×10^{-8}	ND†	
FAP + *M. morganii*	14.1 ± 7.4	0.203 ± 0.017	2×10^{-7}	0.24 ± 0.021	4×10^{-7}
FAP + *P. rettgeri*	5.6 ± 1.2	0.153 ± 0.010	1×10^{-8}	2.4 ± 1.1	6×10^{-7}
Benzylpenicillin + *E. coli* SN01	ND*			0.37 ± 0.08	1×10^{-8}
Benzylpenicillin + *E. cloacae* 908R	1.1 ± 0.09	0.63 ± 0.012	2×10^{-8}	0.73 ± 0.03	1.9×10^{-7}
Benzylpenicillin + *P. rettgeri*	3.2 ± 0.98	0.09 ± 0.005	1×10^{-8}	1.1 ± 0.2	7×10^{-7}

* Difference between α and β not significant.
† A fit could only be achieved by ignoring the initial transient.

the K_m values obtained for the initial and steady-state phases of cefbuperazone hydrolysis (Table 2). Although the value of α depended to some extent on both cephalothin and cefbuperazone concentrations: it was comparable with the values obtained from cefbuperazone hydrolysis (Table 3). The acceleration in rate of cephalothin hydrolysis, while a significant amount of cefbuperazone remained unhydrolysed, suggested that the enzyme is switching between parallel catalytic cycles through isomerization of the free form of the enzyme.

Acylation by slowly hydrolysed substrates

The initial formation of the acyl-enzyme should be monophasic in reactions that branch at the acyl intermediate, while acylation of enzymes where the free form of the enzyme isomerizes can be expected to be polyphasic.

Acylation of the *E. coli* AmpC enzyme by cefbuperazone is clearly biphasic, with approx. 90% of the enzyme reacting rapidly and the remaining 10% more slowly (Figure 5). Acylation was complete before the burst in hydrolysis was over and the rate constants describing the two phases were considerably larger than those characterizing the burst (compare Tables 3 and 5). The time course of acylation was fitted to a double-exponential decrease in activity, eqn. (6):

$$\frac{[A_0]-[a]}{[A_0]} = C_1 \cdot \exp\left[-\left(\frac{kac_1[L]}{Ks_1+[L]}\right)t\right] + C_2 \cdot \exp\left[\left(\frac{kac_2[L]}{Ks_2+[L]}\right)t\right] \quad (6)$$

where $[A_0]$ is the original activity, [a] activity at time t, C_1 and C_2 the amplitudes of the two phases of acylation and kac, Ks are respectively, the rates of acylation and half-saturation constant particular to those phases.

The acylation reaction slows down at pH values greater than 8.5. In the range pH 9–10.5 three phases of reaction were evident in the reaction of the *E. coli* and *E. cloacae* enzymes (Table 5). With the enzymes from *P. aeruginosa*, *P. rettgeri* and *M. morganii*, the reaction was slower at all pH values, and three phases could be distinguished at different substrate concentrations (results not shown).

Isatoic anhydride is an acylating agent that reacts reversibly with the active-site serine residue of β-lactamases (Scheme 2) and

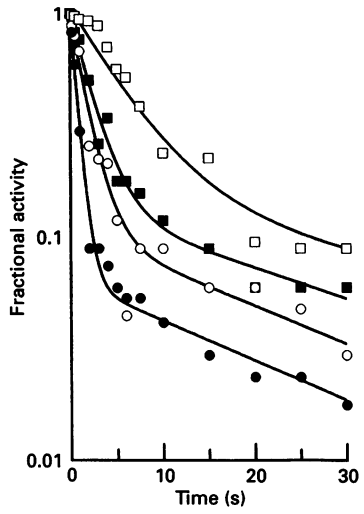


Figure 5 Time course of the reaction between cefbuperazone and *E. coli* SN03 β-lactamase

Cefbuperazone was mixed with enzyme, and loss of activity with nitrocefin was determined as described in the text. The cefbuperazone concentrations were 6.25 (□), 12.5 (■), 25 (○), and 50 (●) μM. The values plotted are the means for three experiments; the curves are calculated for a double-exponential decay of activity (eqn. 6).

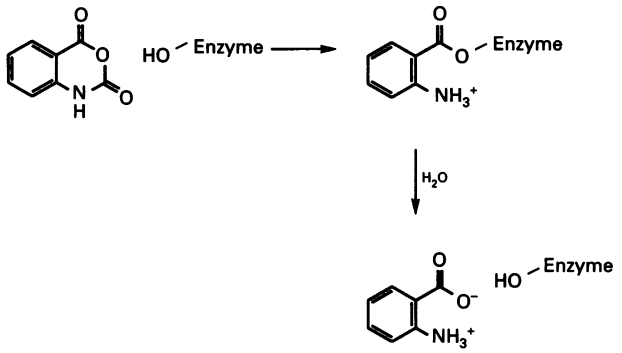
can be used as a probe of the reactivity of this group (M. G. P. Page, unpublished work).

The inhibition of many class C enzymes is apparently biphasic at pH 7.0, and the loss of activity is well described by a double-exponential decay (Figure 6). The formation of anthranoyl-enzyme has a similar time course at the same pH (Figure 7), but three exponential phases can be distinguished because of the better resolution of this method (Figure 7). The reaction with isatoic anhydride slows down at higher values of pH, and three phases can also be resolved in the inhibition time course (Figure 8). There would appear to be three reactive conformations of the free form of the enzymes reacting independently (Scheme 3). Here E_F, E_M, E_S represent three different populations of enzyme that result rapidly (E_F), less rapidly (E_M) and slowly (E_S) with isatoic anhydride. The respective acylation rates are indicated by kac_1, kac_2 and kac_3. The observation of three phases of acylation

Table 5 Kinetic parameters describing the acylation reaction

The rate of acylation was determined by assaying residual nitrocefin hydrolysis as described in the text, except for the reaction with isatoic anhydride at pH 8.5, where the reaction was also followed by monitoring the formation of anthranilate using the absorption increase at 340 nm; the kinetic parameters determined for the reaction are shown in parentheses. The reaction conditions were those described in the text, except for the reaction of the enzyme (1 μM) and isatoic anhydride (200 μM), where the buffers were 0.1 M $NaHCO_3$/0.1 M Na_2CO_3 mixtures of appropriate pH. E_F, E_M and E_S refer to reaction in fast, medium and slow phases of acylation and are identified with the forms in Scheme 3. The limiting rate of acylation, k_{on}, and the half-saturation constant, K_s, were determined from direct linear fits to the Michaelis–Menten equation. ND indicates that this phase of reaction could not be resolved under these conditions.

	E_F			E_M			E_S		
Substrate	kac_1 (s^{-1})	K_s (μM)	Amplitude	kac_2 (s^{-1})	K_s (μM)	Amplitude	$10^3 \times kac_3$ (s^{-1})	K_s (μM)	Amplitude
Cefbuperazone									
E. coli SN01, pH 7	ND	ND	ND	20±3	430±16	0.9±0.1	210±50	500±20	0.10±0.03
pH 8	ND	ND	ND	33±4	500±51	0.88±0.06	350±60	720±45	0.12±0.04
pH 9	60±7	420±19	0.20±0.08	35±6	760±85	0.63±0.09	500±11	715±68	0.17±0.05
pH 10	40±8	410±31	0.20±0.09	11±2	740±77	0.60±0.08	50±10	730±81	0.2±0.09
Ceftriaxone									
E. coli SN01, pH 7	ND	ND	ND	3.3±0.4	14.7±3.1	1.0±0.2	ND	ND	ND
E. cloacae 908R, pH 7	15.1±29	21.1±27	0.2±0.08	5.3±0.5	18.9±2.1	0.31±0.05	1.200±300	25.6±3.9	0.5±0.1
Isatoic anhydride									
E. coli SN01									
pH 8.5	ND		ND	1.26±0.41		0.30±0.04	400±140		0.41±0.19
	(15.1±3.1)		(0.18±0.04)	(1.6±0.3)		(0.30±0.04)	(500±50)		(0.52±0.05)
pH 9.0	2.15±0.29		0.15±0.03	0.36±0.05		0.31±0.04	11±4		0.46±0.08
pH 9.5	0.61±0.09		0.14±0.03	0.09±0.011		0.32±0.05	0.11±0.03		0.44±0.08
pH 10.0	0.42±0.05		0.12±0.05	0.0051±0.0009		0.28±0.05	0.046±0.008		0.60±0.21



Scheme 2 Reaction of isatoic anhydride with the active-site serine residue of β-lactamase

A similar reaction mechanism has been proposed for the reaction of isatoic anhydride with serine proteinases (Moorman and Abeles, 1982).

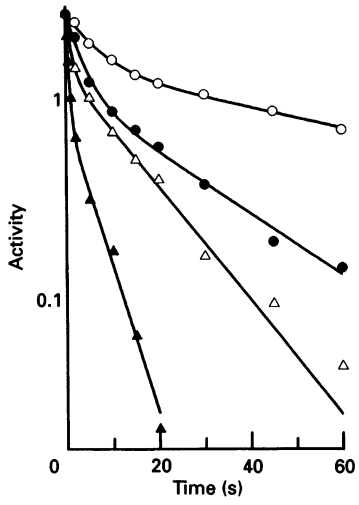


Figure 6 Inhibition of *E. coli* AmpC β-lactamase with isatoic anhydride at pH 7

The assays were performed as described for acylation measurements in 0.1 M sodium phosphate buffer, with 1 μM enzyme and the following concentrations of isatoic anhydride: 20 (○), 50 (●), 100 (△) and 200 (▲) μM. The values plotted are the means of three experiments; the curves are calculated for a double exponential decay of activity.

parallels the behaviour seen with cefbuperazone and is consistent with the burst kinetics seen with FAP and *C. freundii* β-lactamase (Scheme 1).

Analytical ultracentrifugation

We undertook an investigation of the hydrodynamic properties of the enzymes in order to test the hypothesis that the polyphasic kinetics of acylation discussed above were due to allosteric effects in dimers or higher oligomers of the protein. However, all the enzymes examined were found to be monomeric, and the presence of inhibitors that form long-lived acyl complexes made no significant difference to the size, and little difference to the shape, of the protein (Table 6). Thus changes in the oligomeric state of the proteins cannot account for the polyphasic kinetics, and therefore these must be attributed to different conformations of monomers.

DISCUSSION

Isomerization of the free form of enzymes between different conformational states that have different affinities for substrate (and products) has been demonstrated for both oligomeric enzymes (Halford, 1971; Howlett et al., 1977; Albery and

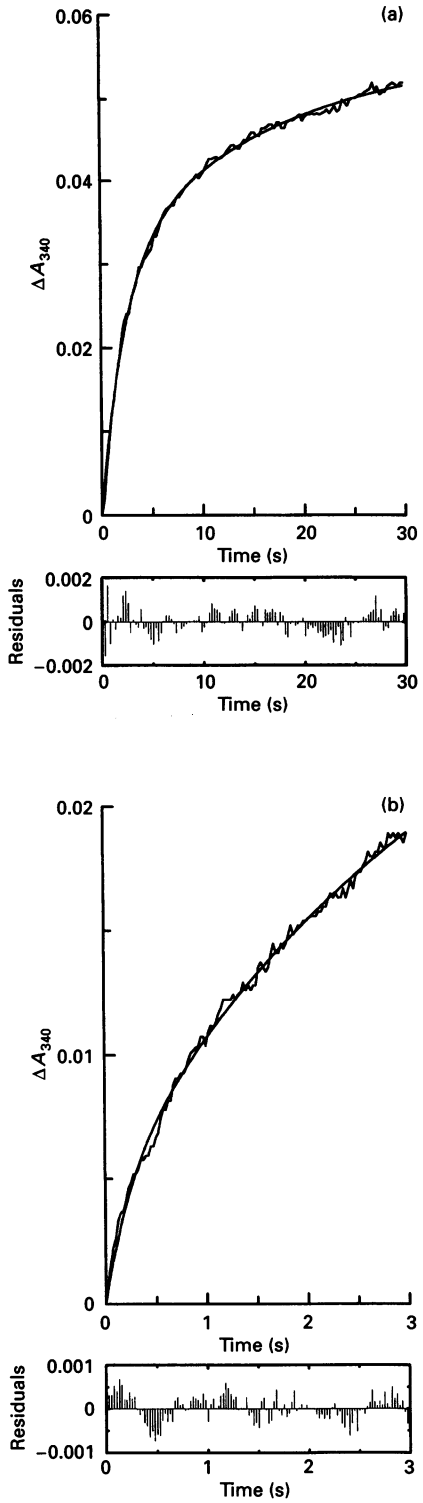


Figure 7 The change in A_{340} occurring during the reaction between *E. coli* AmpC β-lactamases and isatoic anydride

The absorption changes during (**a**) the approach to the steady-state rate of hydrolysis and (**b**) the initial transient, are shown on separate time scales. Each trace is from a single experiment. In both cases the reaction was started by rapidly mixing equal volumes of 1 μM protein and 100 μM isatoic anydride in 0.1 M $NaHCO_3$ at pH 8.5. The smooth curve in (**a**) was calculated using parameters determined by fitting the exponential data to $\Delta A = A_0 \cdot t + A_1(1-e^{-\delta t}) + A_2(1-e^{-\epsilon t})$. The values obtained were $A_0 = 0.0001 \pm \Delta A\ s^{-1}$, $A_1 = 0.03 \pm \Delta A$, $A_2 = 0.032 \pm \Delta A$, $\delta = 0.4 \pm s^{-1}$, $\epsilon = 0.004 \pm s^{-1}$. The smooth curve in (**b**) was calculated using parameters determined by fitting the exponential data to $\Delta A = A_3(1-e^{-\phi t}) + A_4(1-e^{-\gamma t})$. The values obtained were $A_3 = 0.006 \pm \Delta A$, $A_4 = 0.024 \pm \Delta A$, $\phi = 3.6\ s^{-1}$, $\gamma = 0.2 \pm s^{-1}$.

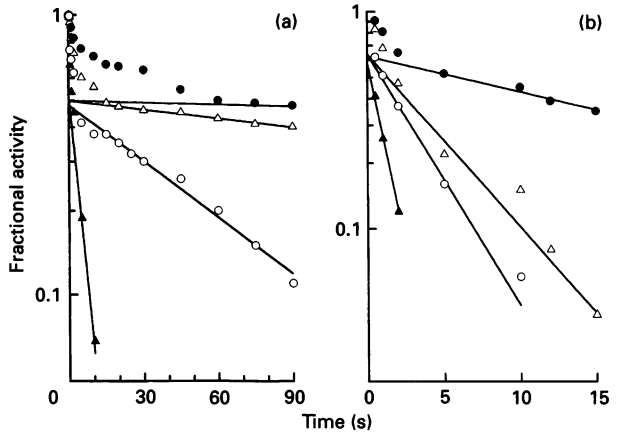
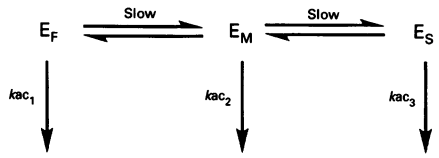


Figure 8 Effect of pH on the rate of reaction between *E. coli* AmpC β-lactamase and isatoic anhydride

(**a**) The reaction was performed in 0.1 M $NaHCO_3/Na_2CO_3$ buffers at pH 8.5 (▲), 9.0 (△), 9.5 (○) and 10.0 (●). Residual activity was determined by measuring nitrocefin hydrolysis at pH 7.0 as described in the text. Values given are the mean of four experiments. The reaction during the slowest phase of acylation is shown by the continuous line, which was calculated according to a triple-exponential decay in activity using the kinetic parameters given in Table 5. (**b**) The data for the initial acylation are replotted according to Ray and Koshland (1961). The reaction during the second faster phase of acylation is shown by the continuous line, which was calculated according to a double-exponential decay in activity using the kinetic parameters for the E_M form of the enzyme given in Table 5.



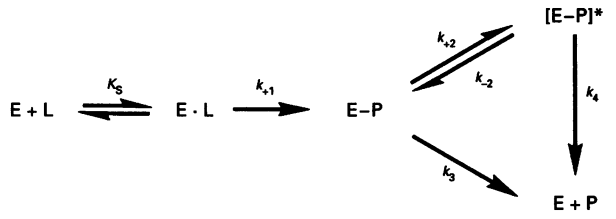
Scheme 3 Acylation of an enzyme existing in three reactive conformations

The rates of interconversion must be slow relative to the rates of acylation (indicated by kac_1, kac_2 and kac_3; Table 5) in order for the reaction to be detected (Rakitzis, 1984).

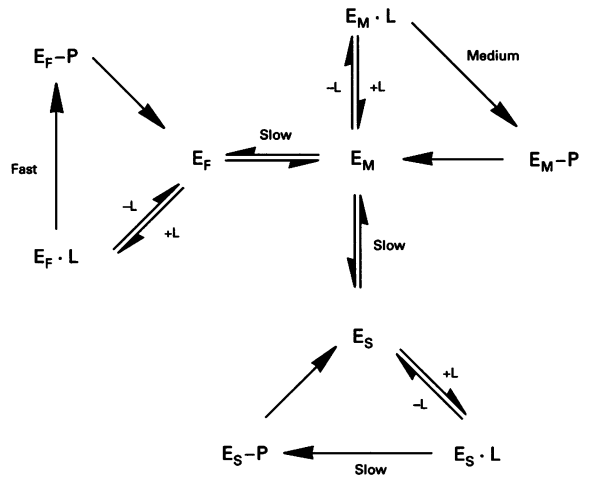
Table 6 Hydrodynamic properties of β-lactamases

Enzyme	$s_{20,w}$ No addition	Molecular mass (kDa) No addition	Aztreonam (20 μM)	Sulbactam (1 mM)
E. coli SN01	3.1	39.5	38.6	36.6
P. aeruginosa 18SH	3.1	39.5	36.4	36.4
P. rettgeri	3.25	37.2	36.1	37.2

Knowles, 1986) and monomeric enzymes (Fersht and Requera, 1971; Meunier et al., 1974; Storer and Cornish-Bowden, 1977). Isomerization of enzyme–substrate complexes is also well-documented [see Morrison and Walsh (1988) for a comprehensive selection of examples] and has been proposed to explain the burst kinetics of β-lactamases [Waley (1991) and references cited therein]. Isomerization of both the free form and the enzyme–substrate complex is integral to allosteric regulation (Kirschner,



Scheme 4 The branched pathway, involving isomerization of the acyl-intermediates, that has been proposed to explain the burst phenomenon in β-lactamase kinetics (Waley, 1991)



Scheme 5 Reaction scheme for class C β-lactamases in which it is proposed that there are three catalytically competent forms of the enzyme (see also legend to Scheme I)

These forms of the enzyme interconvert relatively slowly with respect to the rate of turnover in each of the catalytical cycles.

1971; Kirschner et al., 1971) and has been proposed to explain the burst kinetics of plant-cell-wall glucosidase (Cheron et al., 1990). It is difficult to distinguish between the mechanism in which only the free forms isomerize and mechanisms in which both free forms and bound forms isomerize using steady-state kinetic analysis (Ricard et al., 1974; Neet and Ainslie, 1980; Persaud et al., 1986). Therefore the principal objective of the experiments discussed here has been to establish whether the branched mechanism involving only isomerization of the acyl intermediate is sufficient to explain the kinetics of the burst reaction observed with class C β-lactamases.

The minimum reaction scheme for β-lactam hydrolysis involves binding to form a non-covalent complex, reaction to form the acyl–enzyme complex, hydrolysis of the acyl intermediate and release of the product (Coulson, 1985; Joris et al., 1988; Galleni et al., 1988; Charnas and Then, 1988; Matagne et al., 1990). Such a mechanism can give rise to a stoichiometric burst (that is, a burst having an amplitude no greater than the amount of enzyme) in β-lactam cleavage if the rates of hydrolysis of the acyl intermediate or release of product are lower than the rate of formation of the acyl enzyme. Non-stoichiometric bursts can be caused by inhibition from product or accumulation of the enzyme in a less reactive form. Branched pathways that lead to the formation of a less reactive conformation of the acyl-enzyme (Scheme 4) have been proposed to cause burst phenomena in the reactions of β-lactamases belonging to class A (Kiener et al., 1980; Faraci and Pratt, 1985; Persaud et al., 1986; Fink et al., 1987; Matagne et al., 1990) and class C (Charnas and Then, 1988; Monnaie et al., 1992).

The amplitude of the burst in such a model is determined by the relative rates of the numbered rate constants (Waley, 1991). This model predicts a single phase of acylation, with accumulation of a stable intermediate (the inactivated species, EP*) having the same rate constant as that characterizing the burst (Charnas and Knowles, 1981; Waley, 1991). Both phases should exhibit simple steady-state kinetics that obey the Michaelis–Menten equation (Waley, 1991). The rate constant describing the burst should be dependent on substrate concentration, changing from a limiting low value to a limiting high value as substrate is increased (Waley, 1991). These expectations are not met by the experimental observations.

An alternative explanation for the shift in reactive states is that the free form of the enzyme exists in multiple conformations each of which can undergo reaction with the substrate (Scheme 5).

Such a model can give rise to a burst or a lag in hydrolysis, according to the relative abundance of the different forms and their reactivities. When a burst is observed, its amplitude is determined by the relative rates of reaction in the three parallel catalytic cycles and the rates of the protein isomerizations. When the latter are much lower than the rates of reaction, the enzyme will react as a mixture of three independent species giving three phases of acylation (Rakitzis, 1984). The rate constants for acylation will be higher than those characterizing the burst. In this mechanism, the Michaelis–Menten equation will not necessarily apply, and double-reciprocal plots of steady-state velocity against substrate concentration could be non-linear. In the simple case, with one isomerization reaction, the rate constant describing the burst should decrease with increasing substrate concentration, thus providing a means for distinguishing such a mechanism from one involving isomerization of the acyl intermediate (Waley, 1991). Simulation of reactions depicted in Scheme 5 suggests that this would not necessarily be so. Many combinations of rate constants giving rise to a burst resulted in a constant value for the rate constant characterizing the burst, while others gave rise to such modest increases or decreases with increasing substrate concentration that they could not be expected to be detected experimentally. These predictions fit more closely to the observations described here.

We have observed that the relaxation to the final steady-state rate of hydrolysis is biphasic with several enzymes. This suggests that there are three reactive states of the enzymes. These could represent isomerization of the free form of the enzyme or of any of the reaction intermediates. In acylation experiments we have observed three phases of reaction, which suggests that there are three reactive states of the free form of the enzyme. The experiments involving competition between cephalothin and cefbuperazone strongly suggest that the low rate of hydrolysis is not due to inactivation of the enzyme by isomerization of the acyl intermediate, but rather that the kinetic properties of the free form of the enzyme are being altered by a conformation change induced by reaction with the substrate. The presence of two or more kinetically distinct forms of the enzyme is supported by the observation of non-linear reciprocal plots of substrate-dependence. The most parsimonious explanation of these results is that the class C β-lactamases exist as a mixture of slightly different conformational states that have different kinetic properties and that can slowly interconvert. The interconversion is not

triggered by substrate, but repeated turnover of the enzyme leads to its accumulation in the slowest-reacting cycle.

Preliminary analysis of the crystal structure of the free form of the *E. coli* enzyme has revealed that the loop formed by residues 280–290, which forms one edge of the active site, is rather disordered and may adopt at least two conformations (J. J. Daly, A. D'Arcy, M. G. P. Page and F. Winkler, unpublished work). The geometries of side chains in other positions on the edge of the active site are influenced by the conformation of this loop. Such local conformation changes could easily be the basis of the kinetic differences described here.

This explanation of the burst phenomenon does not preclude the possibility of rearrangement of the acyl–enzyme complex. Indeed, it is quite probable that, in some reactions with inhibitors, protein isomerization and chemical rearrangement both occur and contribute to the overall stability of the acyl–enzyme complex.

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