Efficient catalysis by β -lactamase from *Staphylococcus aureus* PC1 accompanied by accumulation of an acyl-enzyme

Xiaolin QI and Richard VIRDEN*

Department of Biochemistry and Genetics, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, U.K.

The pH- and temperature-dependence of steady-state kinetic parameters for 6- β -(2-furyl)-acryloylamido-penicillanic acid showed it to be a good substrate of staphylococcal PC1 β -lactamase, and the viscosity-dependence of K_m/k_{cat} indicated that steps up to the formation of the acyl-enzyme were partially diffusion-limited. In the pH range 4–9, a pre-steady-state transient blue shift in the UV absorption spectrum of the bound furyl-acryloylamido chromophore was of constant amplitude and decayed to the spectrum of the product with a first-order rate

constant equal to k_{cat} . The spectrum of the isolated denatured acyl-enzyme was similar to that of the methyl ester of furyl-acryloylpenicilloic acid, pointing to non-covalent interactions with the folded protein, possibly associated with the charge on Glu-166, as the source of the blue-shifted spectrum. Taken together, these results point to a rapid acylation and slower deacylation at Ser-70 and imply that ionization of groups affecting enzyme activity at alkaline pH, for which likely candidates are Lys-73 and Lys-234, affect the rate of deacylation.

INTRODUCTION

Crystal structures of β -lactamase (EC 3.5.2.6) from *Staphylococcus aureus* PC1 [1,2] have revealed a catalytic centre containing a complex network of hydrogen-bonded and ionizable groups, and including part of an extensive loop segment (the Ω -loop) of marginal stability [3] which may be significant in substrate-induced inactivation [4]. The structure of an enzyme–inhibitor complex [5] extends the previously proposed catalytic and binding interactions between the protein and the β -lactam ring, and interactions between the penicillin side chain and hydrophobic protein residues have been inferred from modelling; however, a lack of suitable substrate analogues has hampered a more direct crystallographic study of complexes of the active enzyme. An alternative, and an aim of the present work, is to probe the interaction between the enzyme and the bound substrate during catalysis, using a substrate molecule containing a reporter group with optical properties that are sensitive to the environment of the probe. Studies with a rapidly hydrolysed and non-inhibitory substrate, dansyl-penicillin, have shown a transient enhancement of fluorescence [6], characteristic of a non-polar environment of the dansyl side chain in the acyl-enzyme, and acid-quenching of reaction mixtures containing either this substrate or benzylpenicillin at pH 9 have provided evidence for stoichiometric accumulation of the acyl-enzyme, EAc, with $k_{+2} \gg k_{+3}$ (see eqns. 1–4), though other acid-quenching experiments with benzylpenicillin at pH 7 have suggested k_{+2}/k_{+3} to be less than 2 and a small ratio for this value has been proposed to be a requirement for efficient catalysis [7].

$$k_{cat} = \frac{k_{+2} \cdot k_{+3}}{k_{+2} + k_{+3}} \quad (2)$$

$$K_m = \frac{k_{+3}}{k_{+2} + k_{+3}} \cdot \frac{k_{-1} + k_{+2}}{k_{+1}} \quad (3)$$

$$K_{m,acyl} = \frac{k_{-1} + k_{+2}}{k_{+1}} \quad (4)$$

In the present work, we have made use of a substrate containing the furyl-acryloyl group to investigate the relationship between catalytic efficiency and the kinetics of formation and hydrolysis of the acyl-enzyme. The near-U.V. spectrum of this group is known, from resonance Raman studies of a number of substituted furyl-acryloyl-chymotrypsins, to be affected by changes in π -electron polarization which might occur both through a dipole moment set up in the protein and through conformational changes in the acryloyl moiety [8]. The blue shift in λ_{max} , observed under conditions favouring hydrolysis of these derivatives of chymotrypsin, was assigned to the presence of a negative or partial negative charge near the acryloyl carbonyl [9]. 6- β -(2-Furyl)-acryloylamido-penicillanic acid (FA-penicillin) is hydrolysed by PC1 β -lactamase at about the same rate as benzylpenicillin [10] and the reaction is readily followed by a small blue shift in the U.V. spectrum of the product [11]. FA-penicillin forms an acyl-derivative of β -lactamase apparently with $k_{+2}/k_{+3} = 4$, as determined by the fractional recovery of acyl-enzyme after acid-quenching mixtures of enzyme and substrate at pH 6.5 [10]. We now report evidence for a partially diffusion-limited catalytic pathway for this and other good substrates and show that the rapid pre-steady-state change in the spectrum of the furyl-acryloyl group is strongly influenced by the protein environment in the native acyl-enzyme, with kinetics implying that k_{+2} is considerably greater than k_{+3} over a wide range of pH values. A preliminary report of some results has appeared previously [12].

EXPERIMENTAL

Materials

β -Lactamase purified from the supernatant fraction of cultures of *Staphylococcus aureus* PC1 [13] had a specific activity of 115–140 kat/mol with benzylpenicillin. FA-penicillin was

Abbreviations used: FA-penicillin, 6- β -(2-furyl)acryloylamidopenicillanic acid; FA-penicilloic acid, 6- β -(2-furyl)-acryloylamido-penicilloic acid.

* To whom correspondence should be addressed.

from Calbiochem. 6- β -(2-Furyl)acryloylamidopenicilloic acid methyl ester (FA-penicilloic acid methyl ester) was prepared from FA-penicillin by reaction with sodium methoxide as described for benzylpenicillin [10].

Solutions

50 mM Carbonate buffer, pH 9.0, contained 5 mM sodium carbonate/45 mM sodium hydrogen carbonate. 10 mM Phosphate buffer, pH 6.5 and pH 7.0, contained 3.2 mM Na_2HPO_4 /6.8 mM NaH_2PO_4 and 6.1 mM Na_2HPO_4 /3.9 mM NaH_2PO_4 respectively. To prepare solutions of increased relative viscosity, a stock solution containing glycerol or sucrose, at a concentration determined by measurement of the refractive index, was diluted by weight to give the desired relative viscosity [14] in a solution containing 50 mM NaCl, 10 mM sodium acetate and 10 mM phosphate buffer, pH 7.0.

Kinetic experiments

Steady-state kinetic parameters were obtained by analysis of complete progress curves [15] recorded digitally from a Cary model 210 spectrophotometer fitted with a thermostatically controlled cell of light path 10 mm.

A stopped-flow spectrophotometer model SF-3L/SU-40 (Hi-Tech Scientific), light path 2 mm, was used for stopped-flow experiments in the presence of excess substrate. For measurement of time-resolved spectra, progress curves were recorded at each of 21 wavelengths (band width 5 nm) using a stopped-flow spectrophotometer (Applied Photophysics model SX-17MV) with a 10 mm light path. The data were reduced by singular value decomposition and the eight most significant vectors were fitted to the kinetic scheme $A \rightarrow B \rightarrow C$ using global analysis to obtain estimates of the rate constants and to deconvolute the spectra of the species A, B and C [16].

Isolation of the acyl-enzyme

To isolate the acyl-enzyme at 4 °C, 0.14 ml of a β -lactamase solution in 10 mM phosphate buffer, pH 7.0, was mixed with 0.71 ml of a substrate solution in 0.1 M carbonate buffer, pH 9.0, to give 0.14 mM β -lactamase and 0.7 mM FA-penicillin. After 1 s, 0.43 ml of 0.1 M trifluoroacetic acid was added to lower the pH to 2.3. A Superose 12 column equilibrated with an equivalent mixture of buffers and acid was used to separate excess substrate and product from the acyl-enzyme.

RESULTS AND DISCUSSION

Steady-state kinetic parameters

In several respects, the kinetic parameters for FA-penicillin resemble those for benzylpenicillin [10]. At pH 6.5 and 25 °C, k_{cat} was 148 s^{-1} and bell-shaped curves for the pH-dependence of k_{cat} and k_{cat}/K_m in the range pH 3.7–9.0 gave apparent pK values of 3–4 on the acid limb and, on the alkaline limb, 7.8 and 8.4 for k_{cat} and k_{cat}/K_m respectively. In this range of pH, the apparent K_m was independent of the initial substrate concentration, indicating the absence of product inhibition [15]; there was also no time-dependent change in enzyme activity [17], demonstrating the absence of substrate-induced inhibition which is characteristic of such substrates as quinacillin [4]. The linear Arrhenius plot for the temperature-dependence of k_{cat} (Figure 1) gave no evidence of a change in the rate-determining step over the range 4–25 °C. However, the activation energy, $22 \text{ kJ} \cdot \text{mol}^{-1}$, was lower than that observed with benzylpenicillin, suggesting a greater un-

Figure 1 Temperature-dependence of k_{cat} for FA-penicillin

Solutions contained 60 μM FA-penicillin, 5 nM β -lactamase, 50 mM NaCl, 10 mM sodium acetate and 10 mM phosphate buffer, pH 6.5. Error bars represent sample S.D. (three observations).

favourable entropic contribution to the activation free energy for FA-penicillin.

Effects of solvent viscosity

For the acyl-enzyme mechanism (eqn. 1) it follows from eqns. (2) and (3) that the upper limiting value of k_{cat}/K_m , approached when $k_{+2} \gg k_{-1}$, is k_{+1} which, as the rate constant for a bimolecular step, will be limited by the diffusion of enzyme and substrate in the aqueous solvent and thus sensitive to changes in solvent viscosity. The viscosity-dependence of K_m/k_{cat} for benzylpenicillin has been interpreted according to eqn. (5) in terms of partially diffusion-limited catalysis in β -lactamase I from *Bacillus cereus* [7,8] and in two other class A enzymes, including a preparation of the PC1 enzyme with about half the normal catalytic activity [7].

$$\frac{K_m}{k_{\text{cat}}} = \frac{\eta_{\text{rel}}}{k_{+1}} + \frac{k_{-1}}{k_{+1} \cdot k_{+2}} \quad (5)$$

The underlying assumption that a viscous solute influences kinetic parameters solely through effects on the diffusion-limited binding of substrate to enzyme and not indirectly, for example by preferential stabilization of a particular protein conformation, has not always been upheld: an increased k_{cat} has been suggested as the cause of an unexpected absence of a dependence of K_m/k_{cat} upon relative viscosity with the RTM β -lactamase from *Escherichia coli* [7]; a decreased k_{cat} may reflect a reaction limited by proton transfer [19] where k_{cat} is larger than values observed with β -lactamases. The results with PC1 β -lactamase (Table 1) show little effect of sucrose and lower concentrations of glycerol on values of k_{cat} for either FA-penicillin, benzylpenicillin, or the slowly hydrolysed substrate, 6-aminopenicillanic acid, at 4 °C and pH 7. However, k_{cat} values were significantly increased by either sucrose or glycerol at pH 9, effects which are probably comparable with the marked increases in k_{cat} observed with methanol, ethanol and ethylene glycol [10], where the smallest molecule, methanol, was shown not to gain access as a nucleophile in competition with water. The most reliable estimates of k_{+1} and k_{-1}/k_{+2} were therefore obtained where the effects on k_{cat} were small, and the results for FA-penicillin at pH 7 (Figure 2) indicate a reaction partially rate-determined by the diffusion-limited step k_{+1} and with a small value of k_{-1}/k_{+2} . As expected for a poor

Table 1 Effects of increased solvent viscosity on steady-state kinetic parameters

Values of k_{cat} and K_m with sample S.D., the number of observations being shown in parentheses, are given for aqueous solutions with no added viscogen. The relative increase in k_{cat} for a unit increase in relative viscosity ($dk_{\text{cat,rel}}/d\eta_{\text{rel}}$), and the estimated values of k_{+1} and k_{-1}/k_{+2} were derived from weighted linear regression of plots of k_{cat} and K_m/k_{cat} respectively against relative viscosity of the solvent, and are given with the standard errors of the estimates, the number of different values of η_{rel} being shown in parentheses: each datum at a particular value of η_{rel} was an average of 2–6 replicates.

Substrate	Temperature (°C)	pH	k_{cat} (s^{-1})	K_m (μM)	Viscogen	$dk_{\text{cat,rel}}/d\eta_{\text{rel}}$	$10^6 \times k_{+1}$ ($\text{s}^{-1} \cdot \text{M}^{-1}$)	k_{-1}/k_{+2}
FA-penicillin	20	7	133 ± 12 (3)	19 ± 7 (3)	Glycerol	0.11 ± 0.07 (6)	13.5 ± 4.7 (6)	1.1 ± 0.9 (6)
FA-penicillin	4	7	70 ± 7 (3)	12 ± 1 (3)	Sucrose	-0.006 ± 0.018 (14)	9.1 ± 0.6 (14)	0.9 ± 0.2 (14)
FA-penicillin	20	9	4.0 ± 0.2 (3)	8.3 ± 0.2 (3)	Glycerol	0.156 ± 0.026 (11)	2.2 ± 0.5 (11)	4.0 ± 1.3 (11)
FA-penicillin	4	9	1.7 ± 0.2 (2)	2.7 ± 0.4 (2)	Sucrose	0.665 ± 0.049 (14)	-4.2 ± 1.2 (14)	-9.0 ± 2.1 (14)
Benzylpenicillin	20	7	112 ± 6 (3)	12 ± 0.3 (2)	Glycerol	0.237 ± 0.027 (9)	16.4 ± 4.2 (9)	1.9 ± 1.9 (9)
Benzylpenicillin	20	9	3.5 ± 0.1 (3)	0.5 (1)	Glycerol	0.652 ± 0.042 (8)	0.5 ± 0.1 (8)	-0.2 ± 0.6 (8)
Ampicillin	20	7	191 ± 13 (3)	39 ± 6 (3)	Glycerol	0.102 ± 0.021 (9)	6.9 ± 0.8 (9)	0.2 ± 0.2 (9)
6-Aminopenicillanic acid	20	7	5.8 ± 0.3 (5)	171 ± 12 (5)	Glycerol	0.433 ± 0.041 (6)	-0.13 ± 0.01 (9)	-4.4 ± 0.3 (9)
6-Aminopenicillanic acid	20	7	8.2 ± 0.7 (3)	208 ± 89 (3)	Sucrose	0.238 ± 0.018 (7)	-0.5 ± 0.1 (8)	-1.9 ± 2 (8)
6-Aminopenicillanic acid	4	7	3.0 ± 0.1 (2)	136 ± 10 (2)	Sucrose	0.285 ± 0.062 (13)	0.7 ± 0.3 (13)	33 ± 15 (13)
6-Aminopenicillanic acid	20	9	0.4 ± 0.1 (3)	314 ± 10 (2)	Glycerol	0.485 ± 0.050 (18)	-0.0037 ± 0.0007 (18)	-3.1 ± 0.3 (18)

Figure 2 Effect of solvent viscosity on K_m/k_{cat}

K_m/k_{cat} was determined in solutions increased in relative viscosity by either glycerol or sucrose in 50 mM phosphate buffer, pH 7. The conditions were 60 μM FA-penicillin and 6–15 nM enzyme in: $\circ$, sucrose at 4 °C; $\bullet$, glycerol at 20 °C.

substrate, there was little dependence of K_m/k_{cat} for 6-amino-penicillanic acid on solvent viscosity, negative values of k_{+1} and k_{-1}/k_{+2} reflecting a small negative slope of the plot corresponding to Figure 2. Results similar to those for FA-penicillin were obtained for the rapidly hydrolysed substrates ampicillin and, in agreement with published values [7], benzylpenicillin. The effects on K_m/k_{cat} for FA-penicillin at pH 9 were markedly different in glycerol and sucrose, consistent with dominant indirect effects of these solutes; however, in glycerol, where the effect on k_{cat} was smaller, the results suggest a decreased k_{+1} with no great difference in k_{-1}/k_{+2} compared with the values at pH 7. It may be noted that, for a given k_{cat} , K_m and k_{+1} , the ratio k_{-1}/k_{+2} is defined and is therefore independent of k_{+2}/k_{+3} . It has been pointed out [20] that it is sufficient for the value of k_{-1}/k_{+2} to be small in an efficient enzyme, as shown in Table 1 for rapidly hydrolysed substrates, but that, although the contrary has been suggested [7], k_{+2}/k_{+3} may have any value.

It may therefore be concluded that, in spite of indirect effects on catalysis through interaction with viscous solutes, catalysis of the formation of the acyl-enzyme with several normal substrates

of PC1 β -lactamase is efficient, at least near neutral pH values, in the sense of being partially diffusion-controlled.

Stopped-flow kinetics

A marked transient decrease in A_{325} associated with the bound furyl-acryloyl chromophore during enzyme-catalysed hydrolysis has been reported for the hydrolysis of FA-penicillin by PC1 β -lactamase at pH 9 and modelled assuming a slow formation of the Michaelis complex and slow formation of the acyl-enzyme [12]. However, when the initial enzyme concentration was either greater or smaller than that of the substrate, the accumulation of the acyl-enzyme intermediate was complete within about 20 ms, implying k_{+2} to be much larger than k_{+3} . Figure 3 shows stopped-flow results for an enzyme concentration more than 7-fold greater than that of the substrate, where formation of ES is expected to be rapid in comparison with the observable kinetic transients. Similar results were obtained using a 3-fold excess of enzyme and the same substrate concentration, consistent with the absence of a significant contribution from an initial second-order step. The global estimates of the rate constants and their standard errors for the two phases corresponding to k_{+2} and k_{+3} were $244 \pm 2 \text{ s}^{-1}$ and $2.1 \pm 0.1 \text{ s}^{-1}$ respectively, with good agreement between the observed and fitted transients both at the wavelength of maximum amplitude (Figure 3a) and at other wavelengths used to calculate the spectra of the species ES, EAc and P (Figure 3b). A contribution from EP is unlikely, since dissociation of the penicilloic acid product is indicated by the absence of product inhibition noted above. The value of λ_{max} for P was blue-shifted from that of ES by 3 nm, as observed for the product relative to the free substrate [9]. The spectrum of EAc showed a blue-shift of almost 6 nm relative to the product and a 25% decrease in ϵ at λ_{max} .

Formation of the acyl-enzyme partially limited by diffusion is compatible with the present results of viscosity-dependence of K_m/k_{cat} at pH 9, with a smaller apparent value of k_{+1} than at pH 7 (Table 1). This may reflect a rate-determining inter-conversion of the enzyme from a high-pH state to a state capable of binding substrate, plausibly through effects on the stability of the native structure of the Ω -loop. The rate constant for the final transient process was equal to k_{cat} either when the enzyme was present in excess, where the final transient may be expected to have a rate constant equal to k_{+3} , or when the initial substrate

Figure 3 Spectra of transient species during turnover of FA-penicillin

The reaction conditions were: 208 μM β -lactamase; 28 μM FA-penicillin; 50 mM carbonate buffer, pH 9; 4.1 ± 0.1 °C. The data were fitted to the kinetic scheme $A \rightarrow B \rightarrow C$ as described in the Experimental section. (a) Progress curve at 325 nm: full line, experimental; broken line, fitted. (b) Calculated spectra were derived from progress curves recorded at the wavelengths shown (●) for: (—), A; (---), B; (· · ·), C.

Figure 4 Change in A_{325} during complete hydrolysis of FA-penicillin at pH 9

After mixing, solutions contained 29.7 μM enzyme and: (1) 152; (2) 121; (3) 82 μM FA-penicillin. The origin of the time axis is 50 ms after mixing. The temperature was 4.0 ± 0.1 °C.

concentration was greater than that of the enzyme (Figure 4). The kinetics were not affected by the substrate concentration and the linearity was maintained for a large fraction of the reaction,

Figure 5 pH-dependence of k_{+3} and k_{cat}

The temperature was 4 °C. Solutions contained 0.1 M NaCl and either: (pH 3.9–7.8) sodium acetate buffer (total acetic acid + acetate 20 mM) and sodium phosphate buffer (total phosphate 20 mM); (pH 8.3–8.8) 50 mM Tris adjusted with HCl; or (pH 9) 50 mM carbonate buffer. ■, k_{+3} , 100 μM enzyme and 60 μM FA-penicillin; ○, k_{cat} , 1.4–4.7 nM enzyme and 60 μM FA-penicillin. The line was fitted to the relationship: $k_{\text{obs}} = k_{\text{lim}} / (1 + [\text{H}^+]/K_1 + K_2/[\text{H}^+])$.

as expected when the lowest substrate concentration is greater than K_m . Similarly, the kinetics of the linear phase were in agreement with the separately determined steady-state value of k_{cat} . In each experiment, the final phase of increasing absorbance showed an equal amplitude and a first-order rate constant similar to k_{cat} .

The measured amplitude of the pre-steady-state burst was less at pH 8.5 and not detectable below pH 8, consistent with a relaxation time less than the stopped-flow dead-time. This is similar to results with dansyl-penicillin [6], where the kinetics of the process assigned to accumulation of the acyl-enzyme were too fast to be measured at pH values close to neutrality. The amplitude of the final transient, measured in the presence of excess enzyme, was constant in the pH range 4.5–9 with a mean $\Delta\epsilon$ of -3535 ± 523 (S.D.) $\text{M}^{-1} \cdot \text{cm}^{-1}$. The first-order rate constant was closely similar to the value of k_{cat} (Figure 5) and not significantly dependent on the protein concentration, consistent with an approach to saturation by the enzyme. These results suggest a close to stoichiometric accumulation of the acyl-enzyme, with k_{+2}/k_{+3} therefore greater than the value of 4 deduced from acid-trapping at pH 6.5 [10], and greater than the value reported for benzylpenicillin [7]. The yield of acyl-enzyme in an acid-trapping experiment is dependent on the relative rates of the competing processes of enzyme-catalysed acylation and deacylation under the trapping conditions, and the processes of denaturation leading to loss of binding and catalytic function, and this may lead to low yields of trapped acyl-enzyme, compatible with our evidence that acylation is rapid. We therefore conclude that efficient catalysis in the formation of the acyl-enzyme is followed by relatively slow hydrolysis of the acyl-enzyme.

UV absorption of the denatured acyl-enzyme

Small differences in λ_{max} gave a larger change in A_{325} , a decrease relative to the absorbance at λ_{max} representing a blue shift. The results given in Table 2 show that the absorption spectrum of the furyl-acryloyl group in the acid-quenched acyl-enzyme and in the methyl ester of FA-penicilloic acid was unaffected by the addition of urea. The ratio was therefore unlikely to be affected by the presence of urea at neutral pH, which was necessary to prevent refolding and subsequent hydrolysis of the denatured acyl-enzyme. There were similar values for the acyl-enzyme and the

Table 2 UV absorption properties of furyl-acryloyl derivatives

3.5 μ M Acyl-enzyme, containing 0.86 mol of furyl-acryloyl groups per mol of protein, was isolated at pH 2.3 and adjusted by the addition of 10 M urea and 0.1 M NaOH to give the urea concentration and pH shown. Solutions of 52 μ M FA-penicilloic acid methyl ester (FAP-ester), 64 μ M FA-penicillin and 65 μ M FA-penicilloic acid were adjusted similarly. Results are shown as the ratio $A_{325}/A_{\max}$ where $A_{\max}$ was the absorbance at the peak for the furyl-acryloyl group. The error was estimated to be $\pm 5\%$.

Sample	Urea (M)	pH	$A_{325}/A_{\max}$
Acyl-enzyme	0	2.3	0.70
Acyl-enzyme	2.3	2.3	0.71
Acyl-enzyme	2.3	7.0	0.64
FAP-ester	0	2.3	0.70
FAP-ester	0	4.4	0.60
FAP-ester	0	6.2	0.60
FAP-ester	0	7.0	0.59
FAP-ester	2.3	2.3	0.71
FAP-ester	2.3	7.0	0.61
FA-penicillin	0	7.0	0.71
FA-penicillin	0	9.0	0.71
FA-penicilloic acid	0	7.0	0.58
FA-penicilloic acid	0	9.0	0.59

methyl ester of FA-penicilloic acid at both acidic and neutral pH values and the values were more similar to the product penicilloic acid than to the substrate FA-penicillin. In view of the difference between the U.V. absorption properties of the furyl-acryloyl chromophore in the free substrate and the denatured acyl-enzyme, it is unlikely that the single transient with kinetics assigned to k_{+3} contains contributions from the decay of the Michaelis complex. The major cause of the blue-shifted spectrum is therefore from non-covalent contributions of the protein environment in the region of the furyl-acryloyl chromophore in the acyl-enzyme.

The effect of low pH on the acyl-enzyme and other furyl-acryloyl derivatives, corresponding to a red shift in $\lambda_{\max}$ of 3–5 nm, parallels effects observed with several furyl-acryloyl acids, reflecting changes in π -electron polarization. However, the properties of the furyl-acryloyl derivative of β -lactamase at pH 2.3 differ from those of derivatives of chymotrypsin at pH 3, where the spectrum of the acyl-enzyme was red-shifted compared with the denatured protein [8]. It is probable that there is less residual structure in the region of the catalytic centre in β -lactamase at pH 2.3 than in chymotrypsin at pH 3. By contrast, the marked blue shift observed transiently in the active enzyme (Figure 3) parallels a large blue shift in $\lambda_{\max}$ when comparable derivatives of chymotrypsin were brought from pH 3 to an active pH [9]. This was explained by a decrease in π -electron polarization brought about by the presence of a negative charge or a partial

negative charge in the vicinity of the carbonyl group, possibly associated with carbonyl activation by a catalytic base. A comparable mechanism is suggested in the acyl-enzyme derived from FA-penicillin, based on the negative charge associated with Glu-166.

Catalytic mechanism

Glu-166 is a strong candidate as a general base in the deacylation step in the PC1 [1] and other class A β -lactamases and, as recently noted [21], it has also been proposed to act as a general base in the acylation step. The ionization of this group is probably reflected in the acid limb of the pH-dependence for FA-penicillin [10] and the present results suggest no change in the relative contributions to k_{+2} and k_{+3} at acid pH values approaching the pK. The pK for the alkaline limb of the pH profile of k_{cat} for the class A β -lactamase I of *Bacillus cereus* has been shown to have a heat of ionization consistent with a lysine residue [22] and, of other potentially ionizing groups in the catalytic centre, mutagenesis experiments have ruled out a contribution from the conserved residue Tyr-105 in the enzyme from *Bacillus licheniformis* [23]. Of the two plausible candidates, Lys-73 is closer to other catalytic groups than Lys-234, which is well placed to form a salt-bridge with the carboxy group on the β -lactam five-membered ring [1].

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