Kinetic characterization of the acyl-enzyme mechanism for β -lactamase I

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β -Lactamase I catalyses the hydrolysis of penicillins by an acyl-enzyme mechanism. A procedure was developed for determining the rate constants for the acylation and deacylation steps for the good substrates benzylpenicillin and phenoxymethylpenicillin; this depends on determining the fraction of enzyme that is present as acyl-enzyme in the steady state.

INTRODUCTION

β -Lactamases are mechanistically interesting and clinically important enzymes; their action is to catalyse the hydrolysis of the β -lactam ring in antibiotics. Some β -lactamases require metal ions for activity, but most have an active-site serine residue and the key intermediate is then an acyl-enzyme (Scheme 1). An outstanding problem has been the determination of the rate constants for acylation and deacylation for good substrates. We have now solved this problem, for β -lactamase I from *Bacillus cereus*, by measuring the fraction of enzyme that is present as acyl-enzyme under steady-state conditions. Knowledge of this fraction, together with the values of k_{cat} and K_m , enables one to calculate k_{+2} and k_{+3} . On certain assumptions, the rate constants for acylation and deacylation should be comparable for a 'perfect' enzyme, and indeed this is what we find for β -lactamase I.

MATERIALS AND METHODS

Enzyme

β -Lactamase I from *Bacillus cereus* 569H/9 was prepared as described previously (Davies *et al.*, 1974; Baldwin *et al.*, 1980).

Determination of steady-state parameters

The hydrolysis of penicillins by β -lactamase I was monitored at 240 nm in a Cary 219 spectrophotometer; the reaction was carried out at 20 °C in 10 mM-sodium phosphate buffer, pH 7, containing 0.1 M-NaCl and 10 μ M-EDTA. Kinetic parameters were obtained from progress curves (Wharton & Szawelski, 1982), and are the means for at least three determinations. The change in ϵ was 515 M⁻¹·cm⁻¹ for benzylpenicillin and 486 M⁻¹·cm⁻¹ for phenoxymethylpenicillin; these values are based on calibration of the substrates by the alkali uptake, measured in a Radiometer RTS822 Recording Titration System, on enzymic hydrolysis. The corresponding values given by Samuni (1975) were 500 and 405 M⁻¹·cm⁻¹ respectively.

The concentration of β -lactamase I (which was in the range 0.8–2.2 nM) was determined from the first-order rate of hydrolysis of 3 μ M-nitrocefin (K_m 55 μ M); the value of k_{cat}/K_m was 62.3 μ M⁻¹·s⁻¹ at 30 °C in 50 mM-sodium phosphate buffer, pH 7, containing 0.5 M-NaCl and 0.1 mM-EDTA. The concentration of enzyme is given by $K_m \cdot (\ln 2)/(k_{\text{cat}} \cdot t_{1/2})$.

Trapping the acyl-enzyme

The quenched-flow apparatus, modelled after that of Froede & Wilson (1984), comprised three 1 ml Becton Dickinson Plastipak syringes connected via three-way valves. One syringe contained β -lactamase I (usually about 2 μ M) and the second contained substrate (e.g. 3 mM-benzylpenicillin). Thus the initial concentration of substrate in the reaction mixture was 1.5 mM (unless otherwise specified). The third syringe contained 5.4 M-HCl. When the pistons were pushed down the enzyme and substrate were mixed and forced through Teflon tubing (internal diam. 1.1 mm) and thus allowed to react for about 120 ms before the reaction was halted by mixing with acid. The quenched mixture exited the apparatus through a detachable piece of tubing of 0.8 ml volume, which, when emptied after an experiment, provided only the final 27% of the material to leave the syringes, thus ensuring that any material remaining in the apparatus from the previous experiment was swept out. The acidified mixture (0.8 ml) in an Eppendorf tube (Sarstedt, W. Germany) was treated with 0.2 ml of ice-cold 50% (w/v) trichloroacetic acid containing 3.75% (w/v) tungstosilicic acid to precipitate the enzyme. The contents of the tube were mixed by inversion, cooled for 2 min at 4 °C and then subjected to centrifugation. The protein, which remained bound to the tube, was washed with water, and then redissolved in 0.8 ml of 1% SDS in 0.1 M-sodium acetate buffer, pH 4.5.

Determination of the acyl-enzyme

The penamaldate assay for penicilloyl groups was carried out on the SDS solution of the acyl-enzyme in a Cary 219 spectrophotometer as follows. After A_{282} had been recorded, 5 μ l of 1 mM-HgCl₂ was added and the increase in A_{282} was monitored continuously until the absorbance remained constant, which usually took 30–60 min. The change in molar absorption coefficient [typically 18 600 M⁻¹·cm⁻¹; Parker *et al.* (1962) cite values of 18 000–21 300 M⁻¹·cm⁻¹] was measured for each substrate by measuring the change in A_{282} when a known

Scheme 1.

Table 1. Rate constants for the hydrolysis of benzyl- and phenoxymethyl-penicillins by β -lactamase I

The procedures are described in the text, as is the definition of the apparent dissociation constant, K' . Pen G denotes benzylpenicillin and Pen V denotes phenoxymethylpenicillin. The first two entries are control experiments, with either the hydrolysis product of benzylpenicillin (first entry) or β -lactamase I inactivated with 6 β -iodopenicillanate (second entry). The values are given as means $\pm$ S.E.M., with the numbers of experiments in parentheses.

Substrate	Kinetic parameters from quenched experiments			
	$10^3 \times \Delta A_{282}$	$10^{-3} \times k_{+2}$ (s ⁻¹)	$10^{-3} \times k_{+3}$ (s ⁻¹)	K' (μ M)
Penicilloate	0.1 $\pm$ 0.1 (6)			
Pen G*	0.23 $\pm$ 0.28 (6)			
Pen G	6.65 $\pm$ 0.28 (15)	5.0 $\pm$ 0.21	3.93 $\pm$ 0.2	148 $\pm$ 14
Pen V	6.20 $\pm$ 0.33 (14)	5.13 $\pm$ 0.26	4.51 $\pm$ 0.26	135 $\pm$ 10

Substrate	Kinetic parameters from progress curves	
	$10^{-3} \times k_{\text{cat.}}$ (s ⁻¹)	K_m (μ M)
Pen G	2.2 $\pm$ 0.06 (3)	65 $\pm$ 5 (3)
Pen V	2.4 $\pm$ 0.05 (3)	63 $\pm$ 2 (3)

amount, less than 1 molar proportion, of HgCl₂ was added to the acyl-enzyme.

Active-site titration

The stoichiometric reaction of 6 β -iodopenicillanic acid with β -lactamase I (Knott-Hunziker *et al.*, 1979; Loosemore *et al.*, 1980) was used to determine the molar concentration of active enzyme during development of the procedure.

THEORY

The acyl-enzyme mechanism in Scheme 1 leads to:

$$F = 1/[1 + K_m/s)(1 + k_{+3}/k_{+2})]$$

where F is the fraction of enzyme present as acyl-enzyme in the steady state. Since $1/k_{+2} + 1/k_{+3} = 1/k_{\text{cat.}}$, we have:

$$k_{+3} = k_{\text{cat.}}/[F(1 + K_m/s)]$$

Hence k_{+3} , and thus k_{+2} , can be found from measurements of F , when $k_{\text{cat.}}$ and K_m [cf. Froede & Wilson (1984)]. The other parameter found, K' , is defined as $(k_{-1} + k_{+2})/k_{+1}$, is referred to in the text as an apparent dissociation constant, and was found as $K_m(k_{+2} + k_{+3})/k_{+3}$.

The value of $1 + K_m/s$ changes by less than 1% (e.g. from 1.044 to 1.053) during the reaction when the initial concentration of substrate was 1.5 mM, and this change was ignored. At lower concentrations of substrate, the change in F with s_0 was utilized to obtain a value for K_m as follows. A program, based on the simplex algorithm in Press *et al.* (1986), and incorporating Duggleby's (1986) method for obtaining the substrate concentration after time t , was used for experiments at 150, 200, 300, 500 and 800 μ M. Values of K_m and t of 106 μ M and 0.13 s were found, in reasonable agreement with the independently found values of 65 μ M and about 0.12 s.

RESULTS

Tests of the method

β -Lactamase I was inactivated with 6 β -iodo[³H]-penicillanic acid labelled in the β -methyl group (De

Meester *et al.*, 1985), and the radioactive acyl-enzyme was used to show that the protein was (a) quantitatively precipitated and (b) quantitatively extracted by the SDS solution (results not shown).

The concentration of acyl-enzyme was directly proportional to the concentration of enzyme. There was no increase of A_{282} in the penamaldate reaction when the β -lactamase I had been inactivated with 6 β -iodopenicillanic acid, nor when benzylpenicillin was replaced with benzylpenicilloic acid (Table 1). The length of time that the acyl-enzyme was exposed to the solution of HCl used to quench the reaction was about 5 min; when this time was prolonged to 15 min the amount of acyl-enzyme was not decreased, so the acyl-enzyme was adequately stable to acid.

That the concentration of HCl used was high enough to quench the reaction was shown by varying it over a wide range. The fraction of enzyme acylated was independent of [HCl] from (approximately) 1.8 M to 0.018 M.

The changes in absorbance were small (about 0.005), and to attain adequate precision it was necessary to repeat each experiment 12–15 times. The values for the rate constants are fairly sensitive to errors in the value for the fraction of enzyme acylated.

Rate constants for acylation and deacylation

Both the rate constants for acylation and deacylation were high, and of the same order of magnitude (Table 1). The former was slightly the larger, but not by enough for deacylation to be a rate-determining step. The apparent dissociation constant (see the Theory section) was about twice the K_m .

The dependence of k_{+2} and k_{+3} on pH was strikingly similar on the alkaline side (Fig. 1); we do not know whether this was also true for the acid side.

DISCUSSION

At first we tried to find k_{+2} and k_{+3} from the kinetics when the concentration of enzyme was greater than that of the substrate (single-turnover conditions). The change

Fig. 1. pH-dependence of rate constants for acylation and deacylation

The pH-dependence of k_{+2} (●) and k_{+3} (▲) for benzylpenicillin and β -lactamase I is shown. The buffers used were: pH 4.5, 10 mM-sodium acetate; pH 7 and pH 8, 10 mM-sodium phosphate; pH 9, 10 mM-Tris. All the buffers contained 0.1 M-NaCl and 10 μ M-EDTA, and the reactions were carried out at 20 °C.

in A_{232} (Waley, 1974) when benzylpenicillin is hydrolysed was not large enough compared with the absorption due to the enzyme. Nor was the use of the indicator to monitor the release of protons on β -lactam hydrolysis successful; the rate of reaction at a suitable enzyme concentration (of the order of 100 μ M) was too high for measurement in a stopped-flow spectrophotometer. However, the fact that Froede & Wilson (1984) were able to trap an acyl-enzyme from acetylcholine and acetylcholine esterase prompted our trial of this rather direct method. Moreover, the use of the penamaldate reaction is less costly than the use of relatively large amounts of radioactive substrate.

The high value of k_{+2} (5000 s⁻¹) suggests that the condition $k_{-1} \gg k_{+2}$ is unlikely to hold true. This is the condition required for E, S and ES to be present in the same proportions as at equilibrium. Thus K' will differ from k_{-1}/k_{+2} (see the Theory section). Moreover, the finding that the rate of hydrolysis of benzylpenicillin by β -lactamase I is partially limited by diffusion (Hardy & Kirsch, 1984) indicates a high value for k_{+2} . Our conclusion that k_{+3} is only slightly less than k_{+2} for these penicillins, which are good substrates, contrasts with our earlier finding that for cephalosporins, which are poor substrates, $k_{+3} \gg k_{+2}$ (Bickness & Waley, 1985). Thus penicillins and cephalosporins appear to be distinguished here mainly by the value of the rate constant for acylation.

The similar dependence of k_{+2} and k_{+3} on pH (Fig. 1) is most easily explained if the same enzyme groups participate in catalysis of both acylation and deacylation. We have suggested that glutamate-166 might deprotonate

serine-70 in acylation, and water in deacylation (Madgwick & Waley, 1987); this would entail some movement of glutamate-166 from its position in the crystal structure (of the similar staphylococcal β -lactamase) reported by Herzberg & Moulton (1987), and they postulated different roles for the groups in acylation and deacylation. When crystallographic information on β -lactamase I (Samraoui *et al.*, 1986) itself, and the acyl-enzyme, are available it should be possible to resolve these questions.

The fact that k_{+2} and k_{+3} are of the same order of magnitude is interesting in that this is what would be expected for a 'perfect' enzyme, on the assumptions (a) that finite catalytic power is taken to mean that $k_{+2} + k_{+3}$ is constant, and (b) that k_{cat} should be as large as possible.

The determination of individual rate constants is necessary to obtain the full benefit from site-directed mutagenesis experiments in which active-site residues are replaced; for instance, K73R (i.e. the enzyme in which lysine-73 is replaced by arginine) has diminished activity (Madgwick & Waley, 1987), but we do not know whether k_{+2} or k_{+3} (or both) are affected.

Note added in proof (received 11 July 1988)

We have recently found that k_{+2} is similar to k_{+3} in the hydrolysis of good substrates by a second class A β -lactamase, PC1 from *Staphylococcus aureus*.

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