

Trapping the acyl-enzyme intermediate in β -lactamase I catalysis

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Cryoenzymology techniques were used to facilitate trapping an acyl-enzyme intermediate in β -lactamase I catalysis. The enzyme (from *Bacillus cereus*) was investigated in aqueous methanol cryosolvents over the 25 to -75 °C range, and was stable and functional in 70% (v/v) methanol at and below 0 °C. The value of k_{cat} decreased linearly with increasing methanol concentration, suggesting that water is a reactant in the rate-determining step. In view of this, the lack of incorporation of methanol into the product means that the water molecule involved in the deacylation is shielded from bulk solvent in the enzyme–substrate complex. From the lack of adverse effects of methanol on the catalytic and structural properties of the enzyme we conclude that 70% methanol is a satisfactory cryosolvent system for β -lactamase I. The acyl-enzyme intermediate from the reaction with 6- β -(furylacryloyl)amidopenicillanic acid was accumulated in steady-state experiments at -40 °C and the reaction was quenched by lowering the pH to 2. H.p.l.c. experiments showed covalent attachment of the penicillin to the enzyme. Digestion by pepsin and trypsin yielded a single labelled peptide fragment; analysis of this peptide was consistent with Ser-70 as the site of attachment.

INTRODUCTION

The β -lactamases are a class of enzymes that are responsible for the hydrolysis of the lactam bond in β -lactams (penicillins and cephalosporins). The majority of cases of resistance by pathogenic organisms to β -lactam antibiotic therapy are caused by the presence of β -lactamases. The subject of the present investigation is β -lactamase I produced by *Bacillus cereus*, a member of the class A β -lactamases, which includes the homologous enzyme produced by *Staphylococcus aureus* whose high-resolution structure has been reported (Herzberg & Moul, 1987).

From the crystal structure the likely essential catalytic residues are Ser-70, Lys-73, Lys-234 and Glu-166. With the exception of the serine the role of the other active-site residues is unknown, and relatively little is understood concerning the details of the catalytic mechanisms of these enzymes. The covalent involvement of serine has been implicated in the interactions with a number of inhibitors (Knott-Hunziker *et al.*, 1979; Cohen & Pratt, 1980; Cartwright & Coulson, 1980; Fisher *et al.*, 1981; Clarke *et al.*, 1983). Site-directed mutagenesis studies have also shown that Ser-70 is an essential catalytic residue (Dalbadie-McFarland *et al.*, 1982), although the thiol analogue has some catalytic activity (Sigal *et al.*, 1982, 1984). One of the key mechanistic questions has been whether the catalytic mechanism involves nucleophilic attack to form a transient covalent acyl-enzyme intermediate, or whether the reaction is general-acid/general-base-catalysed without a covalent intermediate. Several investigations suggest that both poor and good substrates involve covalent intermediates with various class A and class C β -lactamases (Fisher *et al.*, 1980; Anderson & Pratt, 1981, 1983; Cartwright & Fink, 1982; Cartwright & Waley, 1987; Pratt *et al.*, 1988). The short-lived nature of the putative acyl-enzyme with good substrates has prevented direct demonstration that a covalent bond is formed with Ser-70. In the present study

subzero temperatures were used to advantage to trap and accumulate the acyl-enzyme intermediate and demonstrate its linkage through Ser-70. We have also shown that aqueous methanol cryosolvents have no adverse effects on the enzyme and its function at subzero temperatures. In carrying out this work we also discovered some interesting information concerning the water molecule involved in the hydrolysis of the β -lactam.

MATERIALS AND METHODS

Materials

β -Lactamase I was prepared by the method of Davies *et al.* (1974), some of which was generously given by Dr. Stephen Waley (University of Oxford, Oxford, U.K.), and was homogeneous as judged by SDS/polyacrylamide-gel electrophoresis. Benzylpenicillin was purchased from Sigma Chemical Co., as the potassium salt. Dansylpenicillin (the condensation product of dansyl chloride and 6-aminopenicillanic acid) was synthesized as follows. An aqueous 50% (v/v) acetone solution of 6-aminopenicillanic acid was adjusted to pH 9 before the addition of a concentrated solution of 1.5 equivalents of dansyl chloride in acetone at room temperature. The resulting solution was stirred with constant monitoring of pH, with the addition of appropriate volumes of 3 M-NaOH to maintain pH 8.5–9.0. After 3 h the solution was washed three times with diethyl ether, adjusted to pH 2.5 and saturated with NaCl before being extracted four times with ethyl acetate. The combined ethyl acetate extracts were dried over MgSO_4 before rotary evaporation of the solvent to yield the crude product. The dansylpenicillin was then recrystallized from ethyl acetate/hexane (Cartwright & Fink, 1982). Nitrocefin was generously given by Glaxo Pharmaceuticals. Furylacryloylpenicillin [6- β -(furylacryloylamido)penicillanic acid] was obtained from Calbiochem. [^{14}C]Benzylpenicillin was purchased from Amersham.

Abbreviation used: pH*, apparent pH in cryosolvent.

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Organic solvents and buffer materials were reagent grade. Deionized distilled water was used in the preparation of buffers and cryosolvents. Cryosolvents were stored at -20°C .

Methods

Cryosolvents were prepared on a volume/volume basis, the concentration of the buffer being 0.05–0.1 M. The final ionic strength was made 0.1 with KCl. The pH^* (the apparent protonic activity in aqueous organic solvent) was read directly with a glass electrode between 25 and 1°C . The pH^* value at lower temperatures was calculated from the linear dependence of pH^* on temperature (Maurel *et al.*, 1975). Reactions were initiated by adding portions of stock enzyme in either aqueous buffer or 70% (v/v) methanol at 1°C to precooled solutions of substrate in 3 ml cuvettes. Substrate hydrolysis was monitored by using the spectrophotometric assay method of Waley (1974) at 232 nm, 240 nm or 245 nm for benzylpenicillin, 360 nm for dansylpenicillin, 345 nm for furylacryloylpenicillin and 500 nm for nitrocefin. Corrections were made for the effect of pH, co-solvent and temperature on absorption coefficients. Data were fitted to the integrated Michaelis–Menten rate equation (Samuni, 1975), and values for k_{cat} and K_{m} were calculated from complete progress curves. The pK values for the pH –rate profiles were obtained from fitting the data to the expression:

$$k_{\text{cat}} = \frac{k_{\text{cat}}^{\text{lim}}}{1 + [\text{H}^+]/K_1 + K_2/[\text{H}^+]}$$

Low-temperature apparatus and cryoenzymological procedures were as previously described (Fink & Geeves, 1979).

The acyl-enzyme intermediate was trapped as follows. Furylacryloylpenicillin (100 μl of 30 mM solution in cryo-solvent) was added to 0.9 ml of β -lactamase (10 μM) in methanol/ethylene glycol/water (5:2:3, by vol.), pH^* 5.0, at -40°C and mixed with a vibrating stirrer. After 5 s trifluoroacetic acid (250 μl of 0.5 M solution) was added to lower the pH^* to 2. The sample (1.25 ml) was then loaded on to a mini-gel column (DEAE-Sephadex G-25, 1.3 cm \times 5 cm) and spun for 2 min at 4000 rev./min in a Jouan model GR411 centrifuge at 0°C . The column had previously been equilibrated with 0.4 M-formic acid and spun dry in the centrifuge. The eluent, approx. 1 ml, was collected and concentrated to 0.2 ml volume with a Centricon microconcentrator.

For analytical purposes a portion of the concentrated sample was then analysed by h.p.l.c. with a Beckman gradient h.p.l.c. system equipped with a model 165 detector and a C3 ultrapore RPSC column (4.6 mm \times 75 mm). An acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid [5–45% (v/v) acetonitrile in 15 min] was used, with monitoring at 305 nm (furylacryloylpenicillin) and 280 nm. For isolation of the labelled peptide the concentrated sample was treated with pepsin (β -lactamase/pepsin ratio 10:1 by wt.) at 20°C . H.p.l.c. was used to monitor the digestion. Initially four major labelled peptides were detected. With increasing time of digestion these converted ultimately into a single peak. For collection of the labelled peptides the following gradient elution system was used. Solvent A was aq. 0.1% (v/v) trifluoroacetic acid, and solvent B was acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The gradient began with 5% (v/v) solvent B in solvent A. After 2 min

a linear ramp over 30 min to 45% (v/v) solvent B was followed after 5 min by a linear decrease in solvent B to 5% (v/v) over 18 min. The eluent was monitored at 215 and 305 nm. The sample size was 120 μl . The labelled fractions (305 nm) were combined and dried down with a stream of N_2 .

The dried sample was dissolved in 150 μl of 50 mM-sodium/potassium phosphate buffer, pH 7.8, and digested with trypsin (peptide/trypsin ratio 10:1 by wt.) at 20°C . The reaction was monitored by h.p.l.c. with conditions as above. After 3 h a single labelled peptide was collected from the h.p.l.c. column.

Amino acid compositional analysis was carried out on the peptides after reaction with *o*-phthalaldehyde as described by Schuster & Apfel (1986).

For [^{14}C]benzylpenicillin acyl-enzyme trapping experiments were performed analogously to those with furylacryloylpenicillin with the following modifications. The experiments were carried out on a much smaller scale, 40 μl of buffer, 4 mM in substrate and 7 μM in enzyme, quenched with 10 μl of trifluoroacetic acid solution. The centrifugal column-chromatography step was omitted and 30 μl of the quenched solution was injected directly into the h.p.l.c. system. The enzyme-containing fractions, detected by their absorbance, were collected, and their radioactivity was determined with a liquid-scintillation counter with a standard 2,5-diphenyloxazole/toluene cocktail. Appropriate controls for background values were run.

The reaction of dansylpenicillin with β -lactamase in 70% (v/v) methanol, pH^* 4.8 (sodium formate), at -69°C was monitored by fluorescence. The final concentrations were 22 μM substrate and 22 μM enzyme; excitation was at 285 nm, emission at 515 nm.

RESULTS

The organic co-solvent of choice in cryoenzymology is methanol, owing to the low viscosity and low freezing point of methanol-based cryosolvents. In view of the catalytic efficiency of β -lactamase (e.g. k_{cat} values $>2000\text{ s}^{-1}$) it was expected that quite low temperatures might be needed to stop the turnover reaction effectively. Consequently we chose first to investigate methanol cryosolvents for their suitability.

Effect of methanol on k_{cat} and K_{m}

One of the best means of ascertaining the effect of an organic co-solvent on the catalytic properties of an enzyme is to examine the effect of increasing concentration of the co-solvent on the catalytic parameters for a good substrate. By carrying out these experiments at 0°C the chances of encountering thermal denaturation are decreased. Fig. 1 shows the effect of increasing methanol concentration on k_{cat} and K_{m} for benzylpenicillin and dansylpenicillin. These experiments were performed at 1°C at pH^* 7.0.

There are several points of interest. The enzyme is active to concentrations of at least 90% methanol. (In fact the substrate nitrocefin was completely hydrolysed in the presence of 98.4% methanol at 0°C , although the enzyme underwent slow inactivation!) The value of k_{cat} decreases in a linear manner with increasing methanol concentration to 80% methanol. The additional slight decrease noted at 90% reflects the beginning of the thermal denaturation transition. Increasing methanol

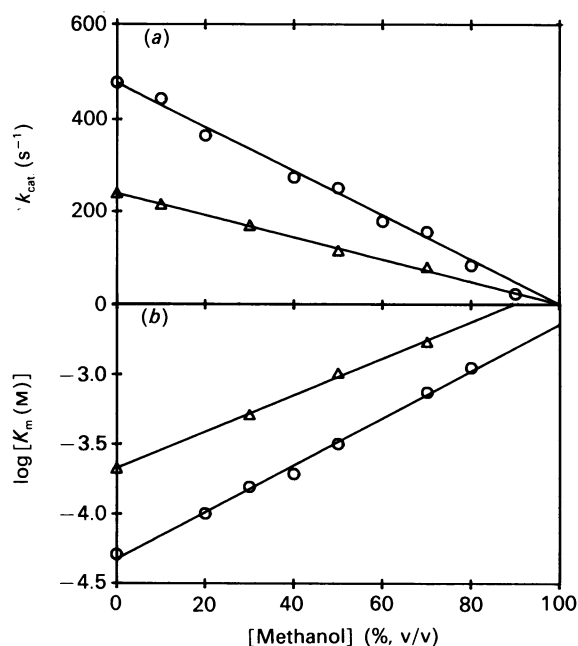


Fig. 1. Effects of methanol on k_{cat} (panel *a*) and $\log K_m$ (panel *b*) in the β -lactamase I-catalysed hydrolysis of benzylpenicillin (O) and dansylpenicillin (Δ)

The continuous lines in panel (*a*) represent the calculated values of k_{cat} , assuming water is involved in the rate-limiting step. Conditions were $pH^* 7.0$ and $1^\circ C$. Buffers were 0.1 M-sodium acetate and 0.1 M-sodium cacodylate.

causes a linear increase in $\log K_m$. There is about a 16-fold increase in going from aqueous to 70% methanol.

Effect of methanol on the pH-dependence of β -lactamase catalysis

In the absence of structural perturbations, cryosolvents may affect the pH-rate profiles of an enzyme-catalysed reaction by virtue of their effects on the pK values of essential catalytic groups (Fink, 1979). In general the pK values of carboxylate groups are raised, whereas those of ammonium groups are little affected. The effect of temperature, however, is just the opposite: carboxylate groups have very low heats of ionization, whereas ammonium groups have large heats of ionization. Thus a decrease in temperature of $50^\circ C$ will typically cause only a 0.5 unit increase in pK of a carboxy group, while a similar decrease in temperature will cause an ammonium group pK to increase by 2 units. Some differential effects with different co-solvents have been noted in this context.

The pH profile of k_{cat} for the β -lactamase-catalysed hydrolysis of benzylpenicillin at $1^\circ C$ in 70% methanol is shown in Fig. 2. The calculated values for the pK values are given in Table 1, along with those for the reaction in aqueous solution at $30^\circ C$, and for the cephalosporin substrate nitrocefin in 70% methanol at $0^\circ C$.

Effect of co-solvent on the structure of β -lactamase

Determining the effect of increasing co-solvent concentration on the intrinsic fluorescence properties of an enzyme has been shown to be a sensitive method for investigating co-solvent-induced structural perturbations (Fink, 1979). Fig. 3 illustrates the effects of methanol on

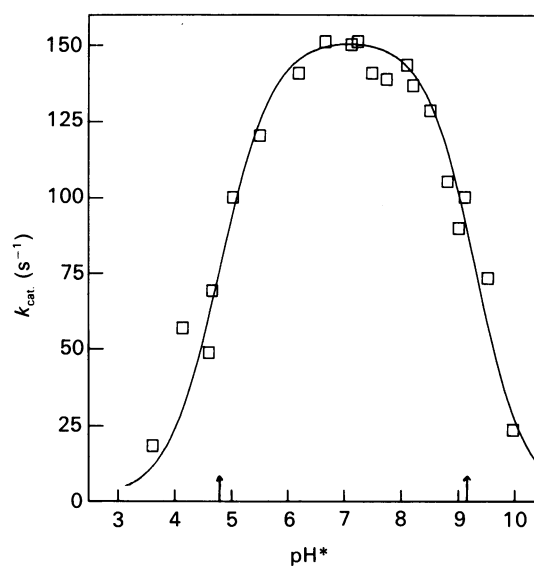


Fig. 2. pH^* - k_{cat} profile for the β -lactamase I-catalysed hydrolysis of benzylpenicillin in 70% methanol at $1^\circ C$

The curve is theoretical, and the arrows indicate the pK values used to calculate the curve. See also the legend to Table 1 for details of experimental conditions.

Table 1. pK values for k_{cat} in the β -lactamase I-catalysed hydrolyses in aqueous and 70% methanol solvent systems

Data for benzylpenicillin in aqueous solution are taken from Waley (1975) and were obtained at $30^\circ C$. Data for benzylpenicillin and nitrocefin were obtained at $1^\circ C$. Buffers used were glycine/HCl ($pH 3.6-4.6$), acetate ($pH 5-7$), phosphate ($pH 7-8$), Tris ($pH 7-9$), morpholine ($pH 8-9$) and glycine/NaOH ($pH 9-10$). The data were corrected for the instability of the enzyme below $pH^* 4.5$, where the enzyme undergoes a pH-induced conformational change to a non-catalytically active form (Goto & Fink, 1989).

Solvent	Substrate	pK_1	pK_2
Aqueous	Benzylpenicillin	4.85	8.6
70% methanol	Benzylpenicillin	4.8	9.25
70% methanol	Nitrocefin	4.2	9.25

the fluorescence emission of β -lactamase ($1 \mu M$) at $1^\circ C$. The effects on the absorbance spectrum are also recorded in Fig. 3(a). Increasing methanol concentrations up to and including 80% cause linear changes in the u.v.-absorbance difference spectra, the fluorescence quantum yield and the fluorescence emission maximum. The break in the plot above 70% methanol (Fig. 3c) reflects a structural perturbation of the enzyme, in this case the onset of thermal denaturation (see below).

In order to determine where the thermal denaturation of the enzyme in 70% methanol began we utilized the intrinsic fluorescence as a probe. The transition begins around $0^\circ C$ and is complete by $25^\circ C$. The T_m is $7.5 \pm 1.0^\circ C$.

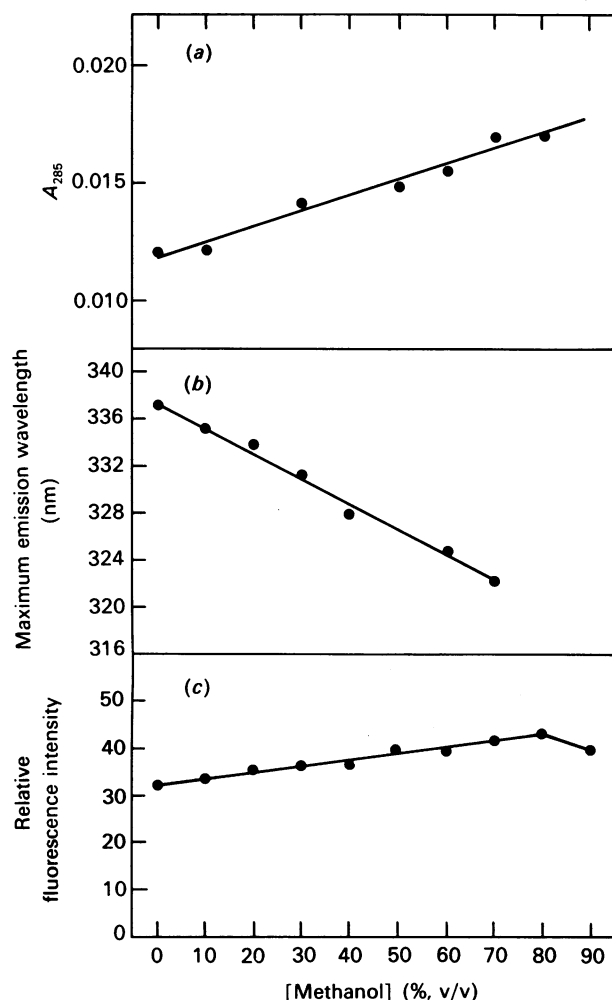


Fig. 3. Effect of methanol on the intrinsic spectral properties of β -lactamase I

Conditions were pH* 6.0 and 1 °C. Buffers were 0.05 M-sodium acetate and 0.05 M-sodium cacodylate. Panel (a) u.v.-absorbance difference spectrum at 285 nm as a function of methanol concentration. Panel (b) fluorescence emission maximum wavelength, with excitation at 260 nm. Panel (c) fluorescence intensity at 337 nm, with excitation at 260 nm.

Effects of subzero temperatures on β -lactamase catalysis

The Arrhenius plots for the β -lactamase-catalysed hydrolysis of benzylpenicillin and dansylpenicillin are shown in Fig. 4 for both aqueous solution and 70% methanol. The values for k_{cat} were determined from initial velocities. For both substrates the Arrhenius plots were linear from 0 to -75 °C, and the energies of activation were similar in aqueous and crysolvent solutions (Table 2). The beginning of thermal denaturation can be observed at the highest temperatures in the experiments in crysolvent.

The effect of temperature on the pK of the alkaline limb of the pH-dependence of k_{cat} was measured in 70% methanol with nitrocefin as k_{cat} substrate. The data are shown in the inset to Fig. 4. A heat of ionization of 44.8 ± 8.4 kJ/mol (10.7 ± 2 kcal/mol) was calculated.

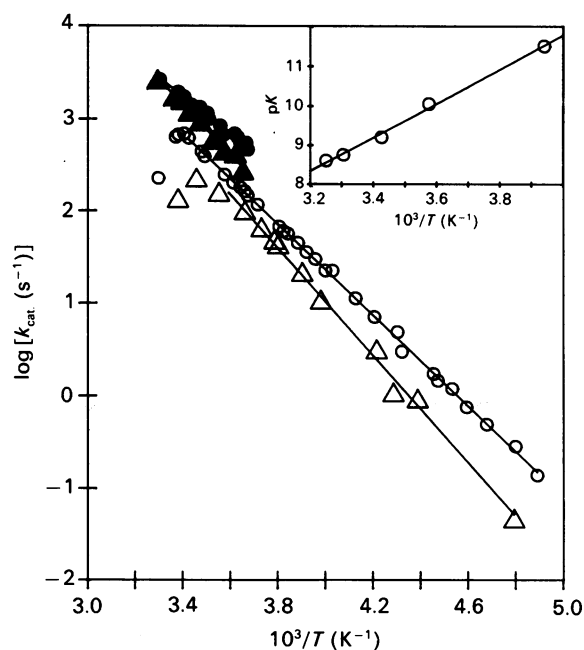


Fig. 4. Arrhenius plots for the reaction of β -lactamase I with benzylpenicillin (\circ and \bullet) and dansylpenicillin (\triangle and \blacktriangle)

The \circ and \triangle symbols represent the data for the reactions in 70% methanol at pH* 6.8 with 0.05 M-sodium acetate buffer. The \bullet and \blacktriangle symbols represent the data for the reaction in aqueous solution at pH 6.8 with 0.05 M-sodium cacodylate buffer. The inset shows the temperature-dependence of the pK for the alkaline limb of the k_{cat} -versus-pH plots for nitrocefin in 70% methanol. The data for the crysolvent experiments at the highest temperatures decrease owing to the onset of thermal denaturation.

Table 2. Energies of activation for k_{cat} for the β -lactamase I-catalysed hydrolysis of benzylpenicillin and dansylpenicillin in aqueous and crysolvent solution

Substrate	Solvent	E_a (kJ/mol)
Benzylpenicillin	Aqueous (pH 6.8)	39.7
	70% methanol (pH* 6.8)	47.3
Dansylpenicillin	Aqueous (pH 6.8)	45.2
	70% methanol (pH* 6.8)	48.1

Trapping the acyl-enzyme

The approach taken to determine whether an acyl-enzyme intermediate could be detected and characterized was the following. Adams *et al.* (1980) have shown that β -lactamase from *S. aureus* is in a non-native conformation at low pH. β -Lactamase I also adopts a non-native, partially folded, conformation at low pH both in aqueous (Goto & Fink, 1989) and aqueous-methanol (D. Joy & A. L. Fink, unpublished work) solutions at moderate to high salt concentrations. We have previously shown that an acid-stable covalent intermediate, presumably an acid-enzyme, is formed in the reaction of β -lactamase I with dansylpenicillin (Cartwright & Fink, 1982). We thus reasoned that if the putative acyl-enzyme intermediate were formed under steady-state conditions

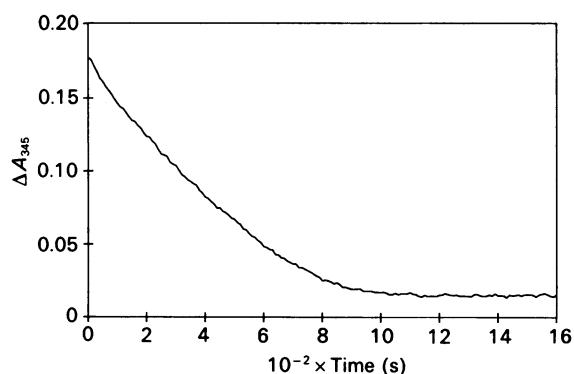


Fig. 5. Absorbance trace of the reaction of β -lactamase with furylacryloylpenicillin at $-40.0\text{ }^{\circ}\text{C}$ at $\text{pH}^* 6.5$

The cryosolvent was methanol/ethylene glycol/water (5:2:3, by vol.). $[\text{E}_0]$ was $1\ \mu\text{M}$ and $[\text{S}_0]$ was $2.2\ \text{mM}$.

at subzero temperature, and that if deacylation were rate-limiting or partially rate-limiting, then quenching the reaction with acid should result in stabilization of the intermediate, provided that the rate of acid-induced conformational change was faster than the rate of deacylation.

Furylacryloylpenicillin was chosen as the substrate because of its chromophore with λ_{max} at 305 nm. The kinetic parameters for the β -lactamase-catalysed hydrolysis of furylacryloylpenicillin in aqueous and methanol/ethylene glycol/water (5:2:3, by vol.) solutions were determined at $0\text{ }^{\circ}\text{C}$ at $\text{pH}\ 7.0$. In aqueous solution k_{cat} was $159\ \text{s}^{-1}$ and K_m was $24.1\ \mu\text{M}$. In cryosolvent the values were $3.0\ \text{s}^{-1}$ and $15.3\ \mu\text{M}$ respectively. Monitoring the reaction by absorbance change at 345 nm in an experiment at $-40\text{ }^{\circ}\text{C}$ (Fig. 5) showed no evidence of intermediate build-up before the turnover reaction. The observed absorbance change corresponds to complete hydrolysis of the substrate. In the trapping experiments the reaction was quenched 5 s after addition of the substrate by the addition of trifluoroacetic acid to lower the pH^* to 2. Excess substrate and product were removed by rapid centrifugal ion-exchange chromatography. The column eluent was analysed by h.p.l.c. (Fig. 6a), which showed the presence of the furylacryloylpenicillin chromophore co-eluted with the enzyme, as well as the presence of unmodified substrate and product. The stoichiometry was calculated to be 0.8:1, indicating (1) that deacylation is rate-limiting, (2) that acylation is fast under these conditions since the steady state was established within 5 s, and (3) that acid-quenching was faster than deacylation.

The trifluoroacetic acid-quenched sample was digested with pepsin and the peptides were separated by h.p.l.c. Initially four furylacryloylpenicillin-labelled peptides were detected. With longer periods of treatment with pepsin these reduced to a single labelled peptide (Fig. 6b). Amino acid analysis indicated this was a large peptide, so subsequent digestion with trypsin was undertaken. Trypsin digestion of each of the four initially labelled peptic peptides gave the same single labelled tryptic peptide (Fig. 6c). Amino acid compositional analysis of this peptide showed the following residues (molar proportions in parentheses) Ala (2), Lys (1), Phe (2), Ser (1) and Thr (1), corresponding to the active-site sequence from residue 66 to 73, with the exception of a missing tyrosine

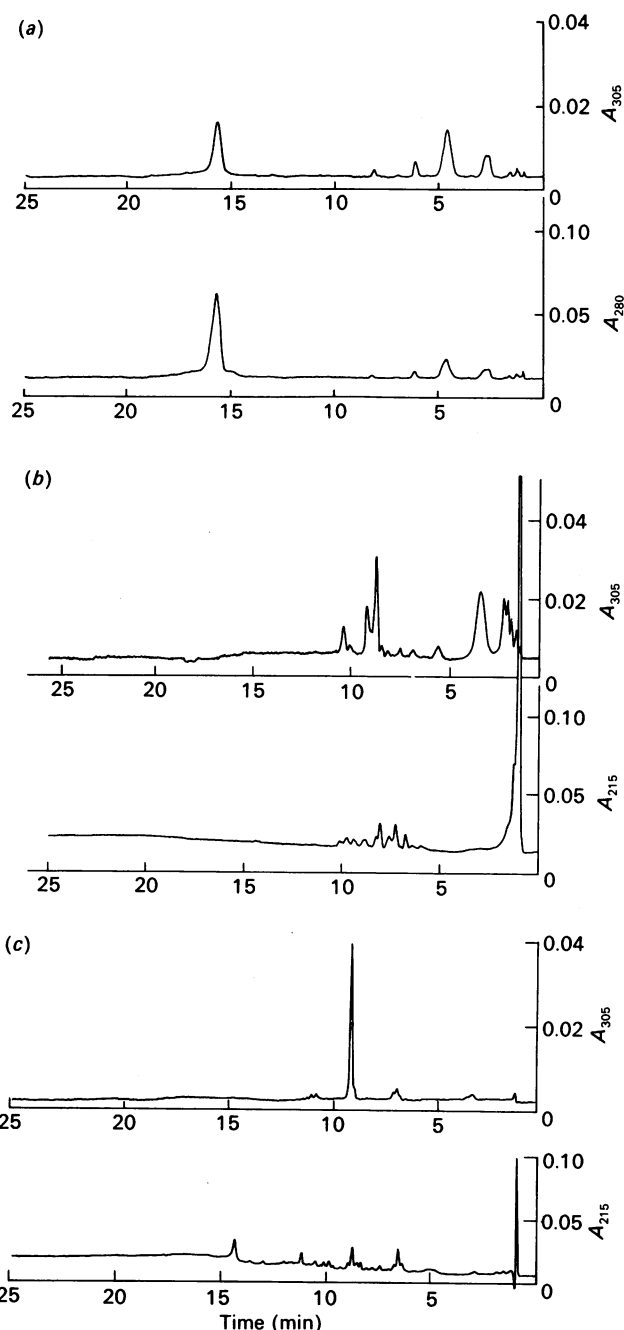


Fig. 6. H.p.l.c. traces of furylacryloylpenicillin-labelled β -lactamase and peptides

Panel (a) shows the results after the initial mini-gel filtration before pepsin digestion. The enzyme is eluted at 16 min, and the substrate and product (and product decomposition products) in the 3–5 min region. Panel (b) shows the elution profile after partial pepsin digestion. The peaks with retention times at 11 and 9 min slowly convert into the peak at 8 min. Panel (c) shows the elution profile after trypsin digestion of the labelled fractions from the pepsin digestion. A single labelled peptide with retention time of 9 min is observed.

residue. The amino acid composition of this peptide is unique to the 66–73 sequence, and we believe that the tyrosine residue may have been destroyed during the amino acid analysis (as is sometimes observed).

When the trapping experiment was carried out at pH 7.0 at 25 °C, rather than at -40 °C (in aqueous buffer), no acyl-enzyme was trapped by the acid quench, as determined by h.p.l.c. analysis (which confirmed that excess substrate was still present). However, if the reaction was carried out at pH 3.5 in aqueous solution (substrate added to enzyme solution) at least 63 % of the acyl-enzyme was trapped.

Similar experiments were performed with [¹⁴C]benzylpenicillin at -40 °C in cryosolvent at pH* 5.0 and at 0 °C in aqueous buffer, pH 7.0. In both cases determination of the radioactivity in the h.p.l.c. eluent indicated no significant increase in radioactivity over the background. We calculate that a maximum of less than 10 % of the enzyme could have been in the form of the acyl-enzyme.

A fluorescence energy-transfer experiment was carried out with dansylpenicillin (Cartwright & Fink, 1982) at -69 °C at pH* 4.8 in 70 % methanol. The rationale was as follows. The emission band of tryptophan overlaps the excitation band of dansylpenicillin, and thus energy transfer will occur when the substrate is bound. If there are changes in the distance or orientation between the donor and acceptor in different enzyme-substrate complexes these should show up as time-dependent changes in the emission, if the tryptophan is excited. This indeed was found to be the case. However, since under the experimental conditions the dansyl group had some absorbance at 285 nm it is not clear if the results actually reflect energy transfer or just changes in quantum efficiency of the enzyme-substrate complex(es). The kinetics of the observed reaction were biphasic, an initial increase in emission with a first-order rate constant of $4.9 \times 10^{-3} \text{ s}^{-1}$, followed by a decrease, with twice the amplitude and rate constant of $2.3 \times 10^{-3} \text{ s}^{-1}$. The spectral trace looks very similar to that reported recently as Fig. 1(a) in Pratt *et al.* (1988) for the reaction between dansylpenicillin and the β -lactamase from *S. aureus* PC1. On the basis of the rate of turnover at higher temperature we attribute the initial increase in fluorescence to the formation of the acyl-enzyme and the subsequent decrease to deacylation. Corroboration for such an interpretation was obtained in experiments in which the substrate concentration was increased and led to a steady-state plateau before the decrease in fluorescence. Interestingly the intermediate was not observed at higher temperatures or higher pH values, nor was any acyl-enzyme detected in trapping experiments at -40 °C. Thus we conclude that the deacylation step is normally not rate-limiting, but becomes so at low pH and low temperature.

DISCUSSION

Cryoenzymology is a potentially powerful method for obtaining information about normally transient intermediates in enzyme catalysis (Cartwright & Fink, 1981). An essential first step in any such investigation, however, is that of ascertaining what effect the cryosolvent has on the catalytic and structural properties of the enzyme. We have previously shown that the types of experiments carried out in the present investigation are a suitable means of accomplishing this (Fink, 1979). In particular sharp breaks in plots of catalytic or structural parameters as a function of co-solvent concentration are indicative of a co-solvent-induced perturbation. It is clear from our

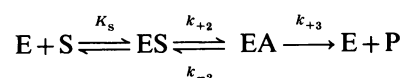
results that β -lactamase I from *B. cereus* is a particularly stable enzyme as far as co-solvent effects are concerned. It is unusual for an enzyme to be essentially fully active in concentrations of methanol as high as 98 % at 0 °C, and to be active in 70 % methanol at room temperature.

Effects of co-solvent on catalysis

The linear decrease in k_{cat} , proportional to the co-solvent concentration, up to and including 80 % methanol at 0 °C (Fig. 1), indicates two main points: (1) no co-solvent-induced structural perturbations affecting catalysis occur over this range of methanol; (2) water is a reagent in the rate-limiting step. This is the simplest explanation of the observation that the observed data points lie on the continuous lines in Fig. 1, which are drawn for the expected decrease in k_{cat} if the concentration of water was involved in the rate-limiting step. An alternative interpretation is that methanol induces a conformational change affecting the catalytic rate in a manner directly proportional to the water concentration; however, the lack of perturbation of the structural probes by methanol suggests this is unlikely. Another conceivable cause could be the increasing viscosity as the co-solvent concentration increases; however, this would only be possible if the rate-limiting step involved the initial substrate-binding step. This may be the case with benzylpenicillin as substrate since the acyl-enzyme trapping experiments suggest that the rate-limiting step with this substrate was acylation at both 0° and -40 °C. This is also supported by the investigation by Hardy & Kirsch (1984) into the effects of viscosity on β -lactamase catalysis. Their results indicate that at pH 7.0 at 25 °C in aqueous solution the rates of reaction for the catalysis of both furylacryloylpenicillin and benzylpenicillin are close to the diffusion-controlled limit, inconsistent with rate-limiting deacylation under their experimental conditions.

There has been considerable uncertainty over the years as to whether class A β -lactamase catalysis occurred via an acyl-enzyme intermediate mechanism, or whether the mechanism was general-acid/general-base-catalysed without a covalent enzyme-substrate intermediate. In conjunction with previous studies, which demonstrated a covalent intermediate in the reaction of good substrates (Anderson & Pratt, 1981, 1983; Cartwright & Fink, 1982), the demonstration of the serine-linked acyl-enzyme intermediate in the present investigation confirms the acyl-enzyme mechanism. The catalytic mechanism may thus be represented by Scheme 1. The observed decrease in k_{cat} with decreasing water concentration (coincident with increasing methanol concentration) is accounted for in terms of this scheme by the rate-limiting step being $k_{+3}[\text{H}_2\text{O}]$.

In the presence of a nucleophile such as methanol, which can compete with water for the acyl-enzyme, one would expect to find transesterification occurring, leading to formation of an additional product, in this case the methyl ester of the penicilloic acid. Experiments to detect the methyl ester formed in the reaction of either benzylpenicillin or dansylpenicillin in 70 % methanol by



Scheme 1.

using both t.l.c. and h.p.l.c. showed that no detectable methyl ester was formed. This indicates that the site at which the attacking water molecule resides in the acyl-enzyme intermediate is inaccessible to methanol. This can be envisaged as involving the water molecule in a site isolated from the solvent, and furthermore a site of geometric and/or electronic constraints that preclude binding of methanol. Given the reported active-site structure of β -lactamase (Herzberg & Moulton, 1987) it is easy to picture a situation in which the key water molecule is completely surrounded by protein and substrate in the acyl-enzyme intermediate. A less likely possibility is that binding of the substrate triggers a conformational change in the enzyme that results in a 'flap' of protein coming down to bury this water molecule and part of the substrate.

The linear increase observed in $\log K_m$ as the co-solvent concentration is increased is commonly observed (Fink, 1979), and is attributed to a hydrophobic partitioning effect. In essence the larger the contribution of hydrophobic interactions to substrate binding the larger the effect (Maurel, 1978; Fink & Cartwright, 1981). If acylation is rate-limiting $K_m = K_s$, whereas if deacylation is the rate-limiting step then $K_m \approx (k_{+3}/k_{+2}) K_s$ and $k_{+3}/k_{+2} < 1$.

The pH-dependence of the β -lactamase-catalysed hydrolysis of benzylpenicillin in aqueous solution has previously been reported at 30 °C; from the data pK values for k_{cat} were given as 4.85 and 8.6 (Waley, 1975). When the reaction was carried out in 70% methanol at 1 °C we found the pK* values to be 4.8 and 9.25. The similar values for the acidic pK suggest either no effect of temperature or co-solvent on the responsible group, or compensating temperature and co-solvent effects. In fact this pK is virtually invariant over the temperature range 0 to -60 °C. If the group responsible for this pK were a carboxy group we would expect no effect of temperature (heat of ionization is approx. 0), but an increase in pK due to the 70% methanol of about 1 unit if the group were exposed to solvent. In conjunction with the active-site residues and these results we conclude that the acidic pK in catalysis is due to a carboxy group that may be buried in the enzyme-substrate complex, and presumably corresponds to that of Glu-166.

The heat of ionization of the group responsible for the alkaline pH-dependence of k_{cat} , as well as the values of the pK, are consistent with a lysine residue being responsible for the decrease in catalytic activity at high pH. On the basis of the conserved lysine residues in the β -lactamases and the active-site residues determined from the X-ray structure (Herzberg & Moulton, 1987), this must be either Lys-73 or Lys-234. If the observed pK* of 9.25 at 1 °C is extrapolated to 30 °C, on the basis of this heat of ionization, a value of 8.6 is obtained, in excellent agreement with the aqueous value at this temperature. That this pK is not perturbed by co-solvent is also consistent with it being due to a lysine residue.

The energies of activation (Table 2) are similar, although not identical, in aqueous and 70% methanol solvents. The small differences can be attributed to slightly different effects of the cryosolvent on the free energy of the intermediates and transition state of the rate-limiting step. The linearity in the Arrhenius plots indicates that there are no changes in the rate-determining step, nor temperature-induced structural perturbations, in 70% methanol over the 0 to -75 °C range.

There is thus no evidence of any adverse effect of methanol on the catalytic properties of β -lactamase I at 0 °C up to and including 70% methanol, and over the 0 to -75 °C range. In view of the fact that the denaturation transition for the enzyme in 70% methanol begins around 0 °C it is likely that cryosolvents of up to at least 90% methanol will be suitable at temperatures considerably below 0 °C.

Effect of co-solvent on structural properties

As mentioned above, it is apparent from both the Arrhenius plots and the intrinsic fluorescence data that the enzyme begins to unfold thermally at temperatures above 0 °C in 70% methanol, pH* 7. The results also indicate that substrate-induced stabilization occurs. The linear or smooth monotonic changes observed in the fluorescence emission or u.v.-absorbance difference spectra (Fig. 3) indicate that no significant structural perturbations are induced by methanol at 0 °C up to and including 70% (Fink, 1979).

In the absence of any detected adverse effects of methanol and subzero temperatures on the catalytic and structural properties of β -lactamase I we conclude that methanol-based cryosolvents are very well suited for cryoenzymological investigations of this enzyme.

Isolation of the acyl-enzyme intermediate

The furylacryloylpenicillin acyl-enzyme trapping experiments reveal a number of interesting points. At the subzero temperatures the rate of acid denaturation must exceed the rate of deacylation, resulting in the quenching of the acyl-enzyme intermediate. The close to 1:1 stoichiometry indicates not only that the catalytic mechanism involves the covalent acyl-enzyme, but that the deacylation rate is either entirely or predominantly rate-limiting.

Analysis of the labelled tryptic peptide indicated it corresponded to the sequence from Phe-66 to Lys-73. On the basis of the crystallographic structure and previous studies with active-site mutants and inhibitors we conclude that the site of acylation was Ser-70. Given the apparent involvement of an essential carboxylate and ammonium group (based on the observed pH-dependence of catalysis as a function of temperature) it is reasonable to propose a catalytic mechanism in which a carboxylate group, presumably from Glu-166, acts as a general base to facilitate nucleophilic attack by O⁻ of Ser-70 on the β -lactam carbonyl group to form a tetrahedral adduct. General acid catalysis by Lys-73 facilitates the cleavage of the lactam bond, leading to formation of the acyl-enzyme intermediate. Deacylation would involve the reverse sequence of events, in which a sequestered water molecule takes the place of the serine hydroxy group.

The lack of detection of the acyl-enzyme in the case of reaction with benzylpenicillin is most probably due to the rate-determining step being acylation for this substrate under the experimental conditions used. This would be consistent with the observations made by Hardy & Kirsch (1984) and recent results reported by Martin & Waley (1988).

The lack of trapped acyl-enzyme with furylacryloylpenicillin when the reaction was carried out at 25 °C at pH 7.0 means one of two possibilities: either the deacylation step is no longer rate-limiting, or the rate of acid denaturation is lower than the rate of deacylation. When

comparable experiments were performed with the *S. aureus* enzyme, in which k_{cat} for furylacryloylpenicillin is about one-tenth that of the *B. cereus* enzyme, the corresponding acyl-enzyme intermediate was trapped at 25 °C (A. L. Fink, A. K. Tan & R. Virden, unpublished work), suggesting that the lack of detectable intermediate in the present case at pH 7.0 at 25 °C was due to the high rate of deacylation. This is consistent with the observation that the acyl-enzyme could be trapped at pH 3.5 at 25 °C, where the rate of deacylation will be much lower than at pH 7. On the other hand, the results reported by Hardy & Kirsch (1984) suggest that the absence of the furylacryloylpenicillin acyl-enzyme in aqueous solution at pH 7.0 is due to acylation being the rate-limiting step under these conditions.

It would appear therefore that the use of subzero temperatures in such trapping experiments may be crucial. We believe that this approach may be of relatively general value in the accumulation and stabilization of covalent intermediates in enzyme catalysis. In cases where the putative intermediate is acid-stable the use of an acid quench should work successfully. In cases where the intermediate is likely to be unstable under acidic conditions quenching with base (if the intermediate is base-stable) or strong denaturant such as guanidinium chloride or SDS may be called for. Preliminary experiments with SDS with the β -lactamase system suggests that it will work satisfactorily. One of the key aspects of such trapping experiments is that the intermediate be accumulated in a close to 1:1 molar ratio with the enzyme. The fact that aqueous-organic cryosolvents frequently cause large increases in K_m means that alternative solvents such as ammonium acetate (Cartwright & Waley, 1987) may be called for in some cases.

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