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Cryoenzymology of Staphylococcal β-Lactamase: Trapping a Serine-70-Linked Acyl-Enzyme†

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Received June 1, 1989; Revised Manuscript Received July 24, 1989

ABSTRACT: Various cryosolvents were investigated for their suitability in cryoenzymological experiments with β-lactamase from *Staphylococcus aureus* PC1. On the basis of the minimal effects on the catalytic and structural properties of the enzyme, ternary solvents containing ethylene glycol, methanol, and water were found most suitable. The interaction of β-lactamase with a number of substrates was studied at subzero temperatures. In general, the reaction profiles were similar to those in aqueous solution at above-zero temperatures, with the exception of the slower rates. For cephalosporin substrates, such as PADAC, in which the 3′-substituent may leave to form a more stable form of the acyl-enzyme [Faraci, W., & Pratt, R. (1985) *Biochemistry 24*, 903–910], this intermediate could be readily stabilized at subzero temperatures. At −40 °C the slow rate of deacylation in the reaction with the chromophoric substrate 6β-[(furylacryloyl)amino]penicillanic acid permitted the acyl-enzyme to be stoichiometrically accumulated. This intermediate was then stabilized at low pH with trifluoroacetic acid. Isolation by centrifugal gel filtration, followed by pepsin digestion, gave a penicilloyl-labeled peptide which was isolated by HPLC. Subsequent trypsinolysis of this peptide gave a single labeled peptide, corresponding to the octapeptide surrounding the active-site serine, Ser-70.

β-Lactamases are very efficient enzymes, with turnover numbers of as much as several thousand per second for some substrates. Until recently, investigations of the catalytic mechanism have been hindered by the lack of a high-resolution crystallographic structure. The structure of the enzyme from *Staphylococcus aureus* has now been reported (Herzberg & Moult, 1987). The major role that these enzymes play in resistance to β-lactam antibiotics makes an understanding of their mechanism of action important for the design of mechanism-based antibiotics.

Various investigations of Class A β-lactamases indicate that the catalytic reaction involves the intermediacy of an acyl-enzyme intermediate (Fisher et al., 1980; Anderson & Pratt, 1981, 1983; Cartwright & Fink, 1982; Pratt et al., 1988). Both studies using inhibitors and those in which Ser-70 has been replaced by other residues via site-directed mutagenesis have implicated Ser-70 as an essential nucleophile in catalysis by Class A β-lactamases (Knott-Hunziker et al., 1979; Cohen & Pratt, 1980; Cartwright & Coulson, 1980; Fisher et al., 1981; Clarke et al., 1983; Dalbadie-McFarland et al., 1982; Sigal et al., 1982). Although a number of other potentially essential residues have been identified, either from modification studies or from the structure of the active site, there is little agreement or information regarding the molecular details of the catalytic mechanism.

In the case of β-lactamase from *S. aureus* additional complications may arise from the conformational mobility of the enzyme. For example, a number of penicillins bring about substrate-induced deactivation, a form of reversible inhibition involving conformational effects (Pain & Virden, 1979; Persaud et al., 1986). In addition, intermediate conformational states have been observed under a variety of conditions including low concentrations of denaturant (Robson & Pain, 1976; Creighton & Pain, 1980; Mitchinson & Pain, 1985) and extremes of pH (Carrey & Pain, 1978; R. H. Pain, personal communication).

The short lifetime of enzyme–substrate complexes under normal conditions renders their study difficult. By use of subzero temperatures to slow the catalytic reaction it is possible that intermediates may be accumulated sufficiently to permit some structural information to be obtained (Fink & Cartwright, 1981; Fink & Petsko, 1981). We report here the results from studies aimed at selecting a suitable cryosolvent and demonstrating the presence of an acyl-enzyme involving Ser-70 with a good substrate. There are three main components to this study: the search for a suitable cryosolvent, investigations of the reaction of β-lactamase with various substrates at subzero temperatures, and the trapping and

† This research was supported by grants from the National Institutes of Health (A.L.F) and the Science Research Council and the Wellcome Trust (R.V.).
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0006-2960/90/0429-145$02.50/0

characterization of the acyl-enzyme.

Experimental Procedures

Materials

β-Lactamase from *S. aureus* PC1 was prepared by the method of Robson and Pain (1976). 6β-[(2-Furylacryloyl)-amino]penicillanic acid (FAP),[1] benzylpenicillin, CENTA, and PADAC were purchased from Calbiochem-Behring. Nitrocefin was a gift from Glaxo Group Research. Dansylpenicillin was synthesized as described previously (Cartwright & Fink, 1982). 6-[(2,4-Dinitrophenyl)amino]penicillanic acid was synthesized from 2,4-dinitrofluorobenzene and 6-aminopenicillanic acid by standard methods (Schneider & de Weck, 1966). HPLC-grade methanol from Fisher was used in making methanolic cryosolvents.

Methods

Cryosolvents were prepared on a volume/volume basis, the concentration of the buffer being 0.05–0.1 M. 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 estimated from the linear dependence of pH* on temperature (Maurel et al., 1975). Reactions were initiated by adding aliquots of stock enzyme in either aqueous buffer or cryosolvent at 1 °C to precooled solutions of substrate. Substrate hydrolysis was followed at 235 or 240 nm for benzylpenicillin, 248 nm for ampicillin, 360 or 385 nm for dansylpenicillin, 345 or 325 nm for FAP, 370 or 450 nm for (dinitrophenyl)penicillin, 566 nm for PADAC, 415 nm for CENTA, and 496 or 500 nm for nitrocefin. Corrections were made for the effect of pH, cosolvent, and temperature on extinction coefficients. Values for k_{cat} and K_m were calculated from complete progress curves by the method of Koerber and Fink (1987). The pK values for the pH–rate profiles were obtained from fitting the data to the expression:

$$k_{obs} = \frac{k^{lim}}{1 + [H^+]/K_1 + K_2/[H^+]} \quad (1)$$

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

Effect of Methanol Concentration on Kinetic Parameters for Benzylpenicillin and Ampicillin. The hydrolysis of ampicillin was followed at A_{248} ($\Delta\epsilon_{248} = -128$ M^{-1} cm^{-1}), using 4.9 mM ampicillin and 0.21 μM β-lactamase at 0.3 °C. The hydrolysis of benzylpenicillin was followed at A_{235} ($\Delta\epsilon = -670$ M^{-1} cm^{-1}), using 0.39 mM benzylpenicillin and 52 nM β-lactamase at 1.1 °C.

Dependence of k_{cat} and K_m on pH in Aqueous and Cryosolvent Solutions. Buffers used were sodium formate, sodium acetate, sodium/potassium phosphate, and Tris·HCl. The substrate was benzylpenicillin, 1.0 mM, and the enzyme concentration ranged from 50 nM to 1 μM. The kinetic parameters were obtained from analysis of complete progress curves (Koerber & Fink, 1987).

Stability of β-Lactamase in Cryosolvents. Typically, 0.1 μM β-lactamase was incubated at different temperatures in cryosolvents containing 50 mM sodium acetate or formate, adjusted to the indicated pH* with acetic or formic acid. At various times, samples were removed and assayed with 2 mM benzylpenicillin, either under the same conditions of pH and temperature or at pH 6.5, 0 °C. Thermal denaturation experiments with cryosolvent were carried out as follows. A solution of 0.9 μM β-lactamase was made in 50% methanol/20% ethylene glycol, pH* 6.5, and sodium phosphate/acetate buffer. The sample was placed in a 0.2-mm path length CD cell at −5.0 °C in the thermostated cell block of an AVIV Model 60DS spectropolarimeter. A linear temperature ramp of 0.36 °C/min, in conjunction with monitoring the ellipticity at 222 nm, was used to follow the thermal denaturation transition.

Interactions of β-Lactamase with Substrates at Subzero Temperatures. A typical experiment was as follows: 1.0 mL of cryosolvent and 20% ethylene glycol/50% methanol, pH* 6.5 (50 mM sodium acetate), containing substrate (1.0 mM FAP) were cooled to −38 °C in the spectrophotometer. An aliquot (10 μL) of β-lactamase solution (a 5-fold dilution of the stock solution with the cryosolvent) was added, to give a final enzyme concentration of 0.7 μM. The solution was mixed with a vibrating stirrer for at least 30 s. Data were collected (ΔA_{325}) until no further change in absorbance was observed. For experiments at pH* 4.4 and 4.8 sodium formate was used as buffer. The hydrolysis of nitrocefin was determined from the increase in A_{496}, measured in a 1-cm light path cuvette, assuming the change in molar absorbance to be 1.97×10^4 M^{-1} cm^{-1}.

The difference spectra measured during the β-lactamase-catalyzed hydrolysis of nitrocefin were determined as follows. The reaction mixture contained 1.2 μM β-lactamase and 23 μM nitrocefin in 70% methanol and 50 mM sodium acetate, pH* 6.5. The spectrum recorded at 225 s after initiating the reaction at −78 °C, where the molar ratio of product to enzyme was 0.25, was subtracted from spectra recorded subsequently.

Product Analysis by HPLC. Benzylpenicilloic acid methyl ester was prepared by reaction of 12 mM benzylpenicillin with 38 mM sodium methoxide in anhydrous methanol at 25 °C for 40 min. In the following incubations at 0 °C, methanol was present at the concentration shown. The solution containing 46% methanol was buffered with 50 mM sodium acetate/acetic acid buffer, pH* 6.5, and other solutions contained 50 mM sodium acetate and 25 mM sodium/potassium phosphate buffer, pH 6.5. Incubation conditions were as follows: (A) 2.3 mM benzylpenicillin and 0.16 μM β-lactamase; (B) 1.0 mM benzylpenicilloic acid ester and 1.47 μM β-lactamase; (C) 2.9 mM benzylpenicillin obtained by dilution of the remaining portion of incubation B after 25 min to give 0.75 mM benzylpenicilloic acid ester and 1.1 μM β-lactamase. At selected times, 20-μL samples were analyzed by HPLC using a reverse-phase column (Alltech Associates, 250 mm × 4.6 mm, 10 μm) eluting with 33% (v/v) methanol in 12.5 mM phosphate buffer, pH 7.0, at a flow rate of 1.5 mL/min. The retention times for benzylpenicillin, benzylpenicilloic acid, and the methyl ester of benzylpenicillin were 12.1 (1.0), 2.8 (0.58) and 12.1 (0.75) min, respectively; relative peak intensities are shown in parentheses.

Low-Temperature Trapping of the Acyl-Enzyme. For experiments with FAP, a solution containing 4–5 mM FAP and 10 or 20 μM enzyme in 20% ethylene glycol/50% methanol, pH* 5.0, was incubated at −40 °C for 5 or 10 s before quenching with 0.5 M trifluoroacetic acid prepared in 20% ethylene glycol/50% methanol, pH* 5.0, to give a final concentration of 0.09 M trifluoroacetic acid and final pH* ≤2.2.

For experiments with 6-[(2,4-dinitrophenyl)amino]-penicillanic acid, 21 μM enzyme was incubated with 18 mM

[1] Abbreviations: (furylacryloyl)penicillin or FAP, 6β-[(2-furylacryloyl)amino]penicillanic acid; dansylpenicillin or DNSpen, 6β-(dansylamino)penicillanic acid; (dinitrophenyl)penicillin or DNPpen, 6β-[(2,4-dinitrophenyl)amino]penicillanic acid; PADAC, pyridine-2-azo-4′-(*N′*,*N′*-dimethylaniline)–cephalosporin; OPA, *o*-phthalaldehyde; pH*, apparent pH in cryosolvent; TFA, trifluoroacetic acid; RT, retention time.

substrate in 20% ethylene glycol/50% methanol, pH* 5.0, at −15 °C for 5 min and then brought to pH* <2.2 by the addition of 0.5 M trifluoroacetic acid prepared in 20% ethylene glycol/50% methanol, pH* 5.0.

Rapid Separation of Excess Substrate from Acid-Quenched Enzyme. "Minigel" columns were prepared from 4 mL (settled volume) of a slurry of DEAE-Sephadex G-25 (fine grade) in a 6-mL plastic syringe body. The column was equilibrated with 0.4 M formic acid by several cycles of washing with 1-mL portions of acid followed by centrifugation at 3000 rpm at 4 °C. After the centrifuge was brought up to speed, the centrifuge drive was immediately switched off so that each cycle was completed within 1 min. The quenched enzyme/substrate incubation mixture was applied to the column and centrifuged in the same way. The solution eluted from the column typically contained 60% of the loaded enzyme and a 10-fold molar excess of β-lactam compound.

Protein, peptides, and β-lactam compounds were analyzed on an Altex Ultrapore C-3 (RPSC) column (4.6 mm diameter × 7.5 cm) operated at a flow rate of 1 mL/min. All solutions contained 0.1% trifluoracetic acid. Before use, the column was equilibrated with 5% (v/v) acetonitrile. Except where fractions were to be collected, the following program was used: (a) 5% acetonitrile, 2 min; (b) 5–45% (v/v) acetonitrile, 15 min; (c) 45% (v/v) acetonitrile, 8 min; (d) reversal of gradient c, 15 min. When fractions were to be collected, this program was modified by increasing the time of step b to 30 min. The eluant was monitored at 280 and 305 nm, or at 215 and 305 nm in the case of peptides (see below).

Peptide Fragmentation and Analysis. Pepsin digestion was carried out on the initial eluant from the minigel column. The final pepsin concentration was chosen to be 1/10 that of β-lactamase. Samples were analyzed by HPLC at various times during incubation at 20 °C.

The sample was concentrated 5-fold by ultrafiltration (Centricon 10, 2 °C, centrifuged at 7000 rpm for 45 min) applied to the HPLC system with the more gradual gradient. The labeled peaks were collected for compositional analysis and were also dried down and subjected to trypsinolysis. The dried sample was dissolved in pH 7.8 phosphate buffer and digested with a 10-fold lower (molar) concentration of trypsin at 20 °C. After 3 h, the sample was loaded onto the HPLC and the FAP-labeled fraction collected.

Trapping the acyl-enzyme from FAP at 20 °C was carried out in a similar manner. β-Lactamase, 100 μL of 10 μM solution, was mixed with 800 μL of pH 5.5 acetate buffer, followed by 100 μL of 30 mM FAP. Five seconds after the addition of the substrate the reaction was quenched by the addition of 250 μL of 0.5 M TFA. The remainder of the experiment was as described above.

Amino acid compositional analysis was carried out on the peptides by using the OPA derivatization procedure as described by Schuster and Apfel (1986).

Results and Discussion

Effects of Cryosolvents on k_{cat} and K_m. A number of experiments were performed in order to choose a satisfactory cryosolvent system. Preliminary screening indicated that aqueous methanol and methanol/ethylene glycol systems looked promising. The effects of increasing concentrations of methanol alone, or in the presence of 20% ethylene glycol, on k_{cat} and K_m for the hydrolysis of benzylpenicillin and ampicillin were investigated at 0 °C. These experiments were carried out at pH* 6.5, a region of pH where the kinetic parameters are relatively independent of pH in both aqueous and aqueous methanol solvents (see below). There was up to a 4-fold

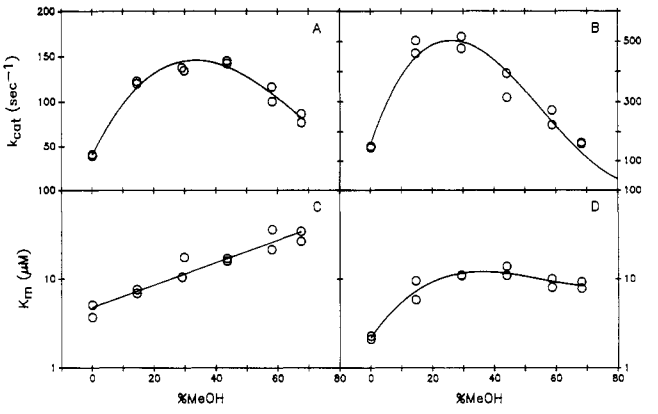

FIGURE 1: Effect of methanol concentration on the kinetic parameters for the β-lactamase-catalyzed hydrolysis of benzylpenicillin (panels A and C) and ampicillin (panels B and D). The pH was 6.5, and the temperature 0.3 °C.

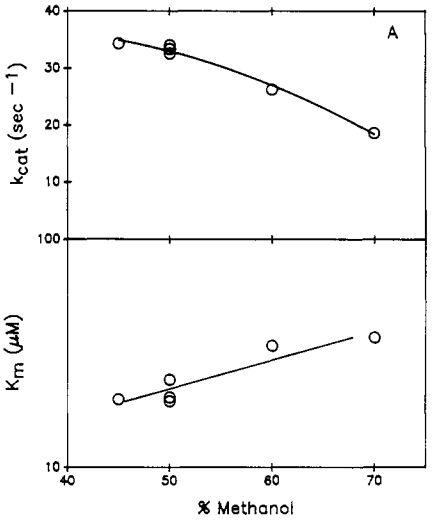

FIGURE 2: Effect of methanol concentration on the kinetics for benzylpenicillin at −15 °C. The hydrolysis of 0.53 mM benzylpenicillin was followed in the presence of 0.16 μM β-lactamase. The pH* was 6.5.

increase in k_{cat} as a function of methanol concentration, with maxima for ampicillin and benzylpenicillin at 35% and 25% methanol, respectively (Figure 1). Although, as described below, the enzyme was not completely stable in some of the methanol solutions, the rates of inactivation were not sufficient to affect the measurement of k_{cat}. In line with this, Figure 2 shows that, at −15 °C where the rate of inactivation was considerably less than at 0 °C, there was a similar decrease in k_{cat} over the corresponding range of methanol concentration. Increasing the ionic strength by making the solution 0.25 M in ammonium acetate had negligible effect on the kinetic parameters but enhanced the protein stability at subzero temperatures: the values of k_{cat} for benzylpenicillin in 0.25 M ammonium acetate/25 mM phosphate buffer, pH 6.5, were 56 and 142 s^{-1} at 0 and 25 °C, respectively; the corresponding values with 50 mM sodium acetate/25 mM phosphate buffer were 52 and 137 s^{-1}. Very similar data to that shown in Figure 1 were obtained in the presence of 0.25 M ammonium acetate and varying methanol concentrations.

Similar shaped curves for the effect of increasing cosolvent on k_{cat} for benzylpenicillin were found with either methanol, ethanol, ethylene glycol, or 20% ethylene glycol/methanol as solvent (Figure 3). We attribute the decrease in k_{cat} at high concentrations of cosolvent to the decreased water concentration, since the decrease is directly proportional to the water concentration, and water is a substrate in the reaction. The

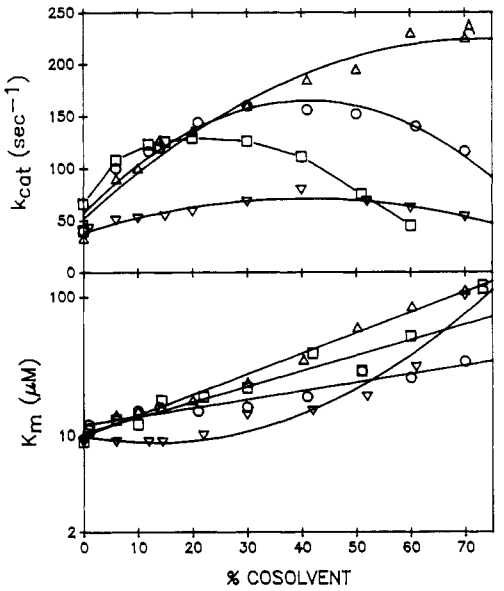

FIGURE 3: Effects of concentration of alcohol cosolvents on the kinetic parameters for benzylpenicillin. The pH* was 6.5, and the temperature 0.3 °C. Cosolvent: (○) methanol; (△) ethanol; (▽) ethylene glycol; (□) 20% ethylene glycol/methanol.

source of the increase in k_{cat} at lower concentrations of cosolvent is more difficult to determine. It is not due to a transfer reaction in which the nucleophilic alcohol competes with water for the acyl-enzyme and leads to formation of a penicilloyl ester (see below) but is most probably caused either by specific binding of the relatively hydrophobic cosolvent molecules to some of the hydrophobic crevices or cavities in the enzyme (Herzberg & Moult, 1987) or by a cosolvent-induced conformational change that increases the catalytic efficiency of the enzyme. However, as noted below, if a conformational effect is involved, it must be rather minor in terms of the protein structure, since probes for structural changes show no significant effects.

With the exception of the ampicillin/methanol and benzylpenicillin/ethylene glycol systems (Figure 1 and 3) the values of K_m increased exponentially with cosolvent concentration in all of the above experiments. Such increases in K_m are commonly observed in the presence of cosolvents and are due to hydrophobic partitioning effects (Maurel, 1978; Fink, 1979). The smaller effect observed with ampicillin reflects the more polar side chain of this substrate and hence the smaller role of hydrophobic interactions in substrate binding.

Dependence of k_{cat} and K_m on pH. The possibility that the effect of cosolvent concentration on k_{cat} was due to a shift in the apparent pK values of catalytic groups was ruled out by measuring k_{cat} as a function of pH in aqueous, 50% methanol, and 20% ethylene glycol/50% methanol solvents (Figure 4) and 70% methanol (not shown). The apparent pK values of 3.7 and 8.0 in aqueous solutions were both increased by about 1 unit in the presence of the cryosolvents, but pH* 6.5 was in the plateau region of all four curves. The effect of pH* on K_m is also shown in Figure 4.

On the basis of the structure of the enzyme and its kinetic properties the most likely residues responsible for the pH dependence of k_{cat} are glutamate (Glu-166) and lysine (Lys-73). Relatively hydrophobic cosolvents would be expected to increase the pK of the glutamate, as observed, and to have little effect on the pK of the lysine. The alkaline pK is increased somewhat in the presence of the cryosolvents, and this may reflect an ionic strength effect. The pH dependence of K_m is U-shaped; the presence of the cosolvents shifts this to

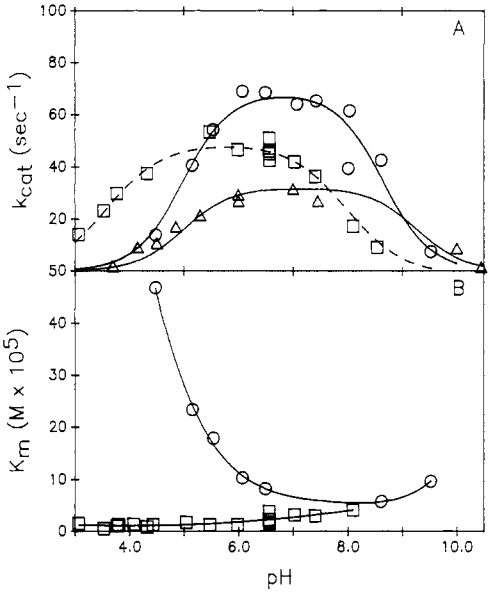

FIGURE 4: Dependence of k_{cat} and K_m on pH in aqueous and cryosolvent solutions. The substrate was benzylpenicillin, and the temperature was 0 °C unless otherwise noted. (Panel A) Data for k_{cat}: (□) aqueous (broken line); (△) 50% methanol (−10 °C); (○) 20% ethylene glycol/50% methanol. (Panel B) Data for K_m: (□) aqueous; (○) 20% ethylene glycol/50% methanol.

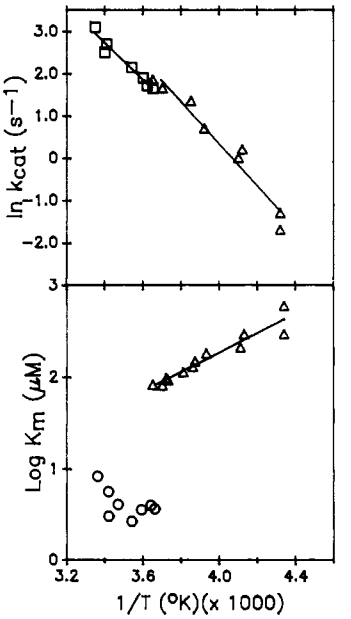

FIGURE 5: Temperature dependence of k_{cat} and K_m. The substrate was benzylpenicillin, and the pH* 6.5. The solvent systems were as follows: aqueous (250 mM ammonium acetate, 25 mM phosphate) (□, ○); 70% methanol with 0.25 M ammonium acetate (△).

higher pH, probably due, at least in part, to the effect of the cosolvent increasing the substrate carboxyl pK.

Temperature Dependence of k_{cat} and K_m. Figure 5 shows Arrhenius plots of k_{cat} for benzylpenicillin at pH* 6.5. In 0.25 M ammonium acetate the energies of activation for aqueous and 70% methanol solutions were 38 and 41 kJ·mol^{-1}, respectively. In 50% methanol/50 mM sodium acetate a value of 51 kJ·mol^{-1} was found. The effect of temperature on K_m is also shown for the hydrolysis of benzylpenicillin in 70% methanol. The linear Arrhenius plots for the aqueous and methanol-containing solvents provide no evidence for a change in the rate-determining step in catalysis, nor for temperature-induced conformational effects, over the temperature range −50 to 30 °C. The similar slopes for the data in aqueous

Table I: Analysis by HPLC of β-Lactamase-Catalyzed Products from Benzylpenicillin[a]

initial conditions	methanol (%)	time (min)	product (mM) penicilloic acid	product (mM) penicilloyl ester
A, 2.3 mM penicillin	2	10	2.4	0.1
A, 2.3 mM penicillin	46	18	1.8	0
B, 1.0 mM ester	8	4	0	1.0
B, 1.0 mM ester	8	22	0	1.0
C, 2.9 mM penicillin + 0.75 mM ester	8	15	3.4	0.6

[a] Incubation conditions (see text): 0 °C, pH* 6.5. β-Lactamase concentrations: (A) 0.16 μM; (B) 1.47 μM; (C) 1.1 μM.

solution and cryosolvent suggests that the cosolvents have no significant effects on the catalytic mechanism.

Analysis of Products by HPLC. The greater nucleophilicity of alcohols compared with water may explain an increased k_{cat} in an alcohol-based cryosolvent if the attack of the nucleophile on an acyl-enzyme is the rate-determining step in the catalytic pathway (Compton et al., 1986). The following experiment (Table I) provides evidence against this interpretation. The product obtained by the action of β-lactamase in the presence of either 1.6 or 46% methanol, or up to 6 mM mercaptoethanol, was identical with authentic benzylpenicilloic acid. Benzylpenicilloic acid methyl ester was not hydrolyzed to benzylpenicilloic acid in the presence of β-lactamase; subsequent addition of benzylpenicillin confirmed that the enzyme remained active. It is unlikely therefore that the increased k_{cat} for hydrolysis of benzylpenicillin in the presence of methanol is associated with a transient formation and subsequent hydrolysis of the methyl ester.

Stability of β-Lactamase in Cryosolvents. Although the pH optimum of β-lactamase is in the neutral pH region, the stability of the enzyme was also examined in cryosolvents at lower pH values in order to determine the feasibility of carrying out reactions with substrates at low values of pH in order to gain in an additional rate decrease.

In 50% methanol, pH* 6.5, the enzyme showed no change in activity at −40 °C for 144 h; however, at −10 °C, 25% of the activity was lost after 3 days. In the same solvent at pH* 4.5, no activity was lost at −40 °C over a 48-h period. In 70% methanol cryosolvent at pH* 6.5, significant loss of β-lactamase activity occurred during incubation at 0 °C; only 5% of the initial activity remained after 24 h. As with 50% methanol the rate of denaturation decreased with lower temperatures; there was negligible loss of activity at −50 °C at pH* 6.5. The enzyme was unstable at pH* 4.5 at all temperatures examined in 70% methanol. However, the presence of ammonium acetate (0.25 M) in the 70% methanol cryosolvent significantly increased the stability of the enzyme, which was then stable at pH* 6.5 at −20 °C.

The stability of the enzyme was markedly improved by the addition of ethylene glycol. In 20% ethylene glycol/50% methanol solutions there was no significant loss of activity at pH* 5.0 or 6.5 during a period of 48 h at −40 °C, and no more than 10% loss of activity at −10 °C at pH* 6.5 over 24 h. At −38 °C there was a 60% loss of activity during 24 h at pH* 4.5; a smaller decrease, approximately 20%, was observed with a solvent containing 30% ethylene glycol/40% methanol. At pH* 4.0 the half-life for loss of activity was 3.5–4 h with either of these cryosolvents. Cryosolvents containing ethylene glycol/methanol are therefore the ones of choice for the higher temperature range and for lower pH values.

Effects of Cryosolvents on Conformational Properties of β-Lactamase. The effect of methanol and ethylene glycol/methanol cryosolvents on the near-UV and far-UV circular dichroism of β-lactamase was determined at 0 °C, pH* 6.5 (Figure 6). In the far-UV the difference spectra showed no differences in ellipticity at wavelengths above 200 nm, indicating that the cosolvents caused no detectable effect on the secondary structure of the protein under the experimental conditions.

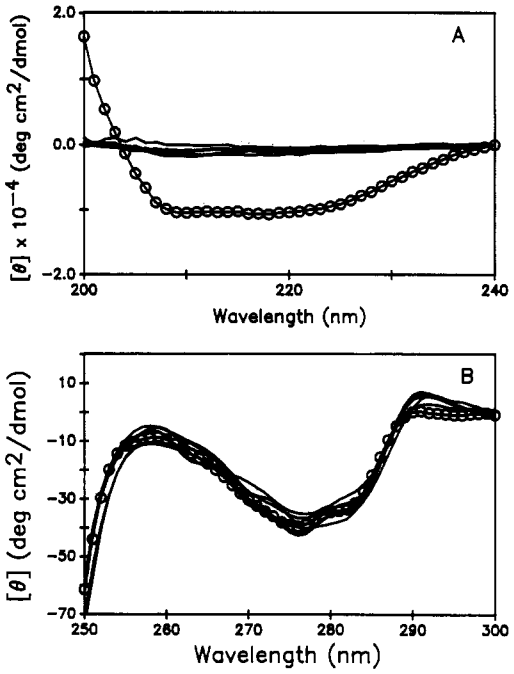

FIGURE 6: Effect of methanol and ethylene glycol/methanol on the circular dichroism of β-lactamase. The temperature was 0.3 °C, and the pH* was 6.5. Panel A shows the far-UV data; panel B represents the near-UV data. In each case the ellipticity of the enzyme in *aqueous* solution is shown by circles. In Panel A the solid lines without symbols represent the difference spectra for 15, 30, and 45% methanol, and for 20% ethylene glycol with 30 and 50% methanol, in which the spectrum of the enzyme in aqueous solution has been subtracted. Within experimental error the spectra for all the solvents were the same. In Panel B the solid lines represent the spectra for these same solvent systems; again within experimental error the spectra are the same as that for the aqueous solution except in the vicinity of 290 nm (see text).

The near-UV CD spectra were examined in a similar manner and also showed no evidence for cosolvent-induced structural effects on the protein. The difference spectra, within the limits of the signal/noise errors, show no evidence for solvent effects on the CD properties of the exposed tyrosine residues with the possible exception of a small increase in ellipticity around 290 nm, attributable to a solvent effect on the electronic properties of the tyrosine rings.

Such solvent effects on exposed tyrosine residues were also apparent in experiments in which the fluorescence emission properties of the enzyme were measured as a function of methanol concentration (Figure 7). Such effects are commonly observed (Fink, 1979). The observed increase in emission intensity of the enzyme in the presence of methanol was virtually the same as the increase observed for *N*-acetyl-Tyr ethyl ester (Figure 7). Similar effects were observed with cryosolvents composed of 20% ethylene glycol with varying amounts of methanol.

Attempts to measure the thermal denaturation transition in the presence of cryosolvent were confounded by the tendency of the unfolded (or partially unfolded) protein to aggregate. The transition was monitored by change in ellipticity at 222 nm. The denaturation transition began at 15 °C and was

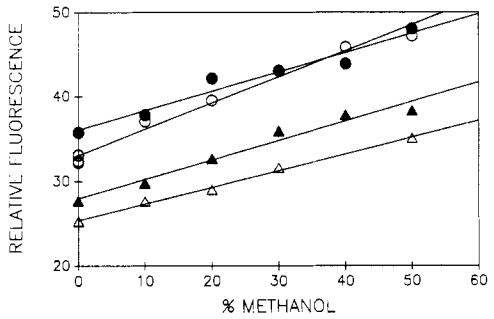

FIGURE 7: Effect of methanol and ethylene glycol/methanol on the fluorescence emission of β-lactamase. The temperature was 1.6 °C for the enzyme (circles) and 3.4 °C for *N*-acetyl-Tyr ethyl ester (triangles), and the pH* was 6.5. Excitation was 277 nm. The open symbols correspond to aqueous methanol solvent systems, and the filled symbols correspond to ternary solvent systems containing 20% ethylene glycol plus the stated concentration of methanol.

Table II: Kinetic Parameters for β-Lactamase Substrates at Various Temperatures

substrate	temp (°C)	solvent	pH	k_{cat} (s^{-1})	K_m (μM)
benzylpen[a]	25	aq[b]	6.5	137	3
benzylpen	0	aq	6.5	38	5
FAP	25	aq	6.5	118	15
FAP	0	aq	6.5	56	7
DNPpen	20	aq	6.5	42	695
DNSpen	0	aq	6.5	40	20
nitrocefin	0	aq	6.5	2.6	1
nitrocefin	0	70% MeOH	6.5	5.1	9
nitrocefin	−15	70% MeOH	6.5	2.7	8
nitrocefin	−53	70% MeOH	6.5	0.017	6
nitrocefin	−30	70% MeOH	4.8	0.078	7
nitrocefin	−35	70% MeOH	4.4	0.027	7

[a] Benzylpenicillin. [b] Aqueous.

complete around 25 °C in 20% ethylene glycol/50% methanol, pH* 6.5.

The results from the CD and fluorescence experiments indicate that the addition of methanol or ethylene glycol/methanol, in the concentration and temperature range examined, causes no signigicant changes in the structure of the enzyme. In conjunction with the experiments on the catalytic properties of β-lactamase we conclude that such solvent systems are satisfactory for studying the enzyme at subzero temperatures, within the constraints of pH*, temperature, and cosolvent concentration determined by the stability tests.

β-Lactamase-Catalyzed Hydrolysis of Substrates at Subzero Temperatures. Several penicillin [e.g., benzyl-, (furylacryloyl)-, and (dinitrophenyl)penicillin] and cephalosporin (e.g., PADAC, CENTA, and nitrocefin) substrates were examined at a variety of different subzero temperatures at pH* 5.0 in 20% ethylene glycol/50% methanol in order to determine which systems were most suited for detailed mechanistic studies. In addition, it was hoped that these experiments might provide some evidence for the accumulation and stabilization of intermediate species at suitably low temperatures. The kinetic traces at subzero temperatures resembled those observed in aqueous solution at ambient temperatures, except for the different time scales, and indicate that the catalytic reaction progresses in a similar way under the cryoenzymological conditions.

The values of k_{cat} and K_m for (furylacryloyl)penicillin (Durkin et al., 1977) in aqueous solution are similar to those for benzylpenicillin (Table II), indicating that it is a very good substrate for this enzyme. At −38 °C in 20% ethylene glycol/50% methanol, pH* 5.0, we obtained values of k_{cat} = 0.08 s^{-1} and K_m = 90 μM. This substrate seemed well-behaved in reactions carried out at subzero temperatures, had a substantial

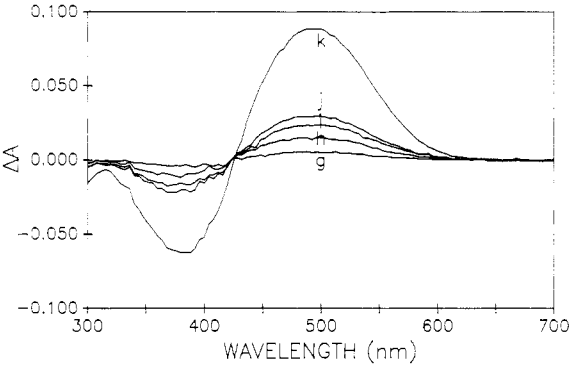

FIGURE 8: Difference spectra during the β-lactamase-catalyzed hydrolysis of nitrocefin at −78 °C in 70% methanol. The reaction mixture contained 1.2 μM β-lactamase and 23 μM nitrocefin. The pH* was 6.5. The spectrum recorded at 225 s, where the molar ratio of product to enzyme was 0.25, was subtracted from spectra recorded subsequently. Moles of product per mole of enzyme: trace g (0.6 h), 0.43; trace h (1.4 h), 0.82; trace i (2.3 h), 1.2; trace j (3.0 h), 1.5; trace k (16.8 h), 3.9.

absorbance change associated with the reaction, and had a reasonably low K_m, allowing for substrate saturation at subzero temperatures.

The catalytic reaction with (dinitrophenyl)penicillin showed clean Michaelis–Menten kinetics in contrast to the reaction with β-lactamase I from *Bacillus cereus*, in which the substrate acts as a reversible suicide inhibitor (A. K. Tan and A. L. Fink, unpublished results). In 20% ethylene glycol/50% methanol, pH* 5.0, −38 °C, the value of K_m was too large (>10 mM) to allow the separate determination of k_{cat} and K_m. A value of k_{cat}/K_m = 5.5 M^{-1} S^{-1} was calculated by using $\Delta\epsilon$ = 173 M^{-1} cm^{-1}.

The β-lactamase-catalyzed hydrolysis of nitrocefin was examined in a number of cryosolvents, at various pH values, over the 0 to −78 °C range. Table II shows kinetic constants for the hydrolysis of nitrocefin in aqueous solution and in 70% methanol. The results at pH* 6.5 showed a temperature dependence similar to that with benzylpenicillin. After addition of β-lactamase to nitrocefin in 70% methanol/sodium acetate buffer, pH* 6.5, at −78 °C there was no evidence of a rapid burst or of a delay in the increase in absorbance at the wavelength (496 nm) characteristic of the product. Difference spectra calculated by subtraction of a spectrum recorded at a time when little product had been formed (225 s) are shown in Figure 8. There was no indication of any species with a spectrum differing from that of the substrate or product. The reaction rate was sufficiently slow under these experimental conditions that a single turnover took almost 2 h! There was also no evidence for the accumulation of an intermediate during the hydrolysis of nitrocefin in 20% ethylene glycol/50% methanol at pH* as low as 4.

With the β-lactamase from *S. aureus* the hydrolysis of PADAC shows complex kinetics due to the formation of the 3′-*exo*-methylene form of the the acyl-enzyme (Faraci & Pratt, 1984, 1985). Such behavior was also observed in the presence of cryosolvent and at subzero temperatures. In fact, the rate of hydrolysis of the *exo*-methylene form of the acyl-enzyme became so slow at lower temperatures that the enzyme was easily accumulated stoichiometrically in this form. In aqueous solution at 0 °C there were about 10 turnovers during the buildup of the *exo*-methylene form of the acyl-enzyme. In 70% methanol under similar conditions there were about 30 turnovers; this was due to the fact that the rate of hydrolysis of the *exo*-methylene form of the acyl-enzyme is faster (about 7-fold) in the presence of the methanol. The rate of turnover was negligible at −79 °C in 70% methanol (0.21 mol of

product/mol of enzyme after 14 h), suggesting that an intermediate was accumulated. The reactions with PADAC will be reported in more detail elsewhere. Similar results were obtained with CENTA, another cephalosporin with a good leaving group at the 3′-position (Jones et al., 1982).

Trapping the Acyl-Enzyme at Subzero Temperatures. The above results suggested that (furylacryloyl)penicillin would be best suited for attempts to trap the putative acyl-enzyme at subzero temperature since its relatively low value of K_m would permit saturating substrate concentrations. By the same token, (dinitrophenyl)penicillin, with its very high K_m, could serve as a control for any nonspecific modification of the enzyme in the trapping experiments discussed below.

The strategy for these experiments was as follows. If the deacylation step were rate-limiting (or even partially rate-limiting), then with saturating substrate concentrations the majority of the enzyme would be in the form of the acyl-enzyme under the low-temperature steady-state conditions. At suitably low temperature the rate of breakdown of the intermediate would be quite slow. Previous investigations have shown that β-lactamase adopts a nonnative, noncatalytically active conformation at pH values below 3 (Carrey & Pain, 1978) and that this is also true in the presence of methanol (D. Joy and A. L. Fink, unpublished results). Depending on the exact conditions (pH, temperature, ionic strength, cosolvent concentration), this process can be quite fast even at subzero temperatures. Thus, if the pH was rapidly dropped after the steady state was established at the low temperature, the acyl-enzyme intermediate might convert into the nonactive conformation at a rate faster than the slow rate of deacylation. Previous studies on the putative acyl-enzyme intermediate have shown that it was relatively stable at low pH (Cartwright & Fink, 1982).

In a typical trapping experiment (furylacryloyl)penicillin (5 mM) was mixed with β-lactamase (20 μM) in 20% ethylene glycol/50% methanol, pH* 5.0 at −40 °C. After 5 s the reaction was quenched by the addition of TFA to bring the pH* to 2. After removal of the majority of the substrate and product by ion-exchange chromatography, the eluant was analyzed by HPLC. As shown in Figure 9A the furylacryloyl chromophore coeluted with the enzyme. Since the chromatographic conditions are such that the protein should be unfolded, this indicates a covalent bond between the (furylacryloyl)penicillin and the enzyme. On the basis of the extinction coefficient of the furylacryloyl chromophore we calculate a 0.8:1 stoichiometry between FAP and the enzyme.

Slow loss of the chromophore was noted on storage of the acyl-enzyme at pH 2, 4 °C; for example, after 18 h about 25% of the label had been released. If the acyl-enzyme sample was adjusted to pH 5.8, incubated at 18 °C for 30 min, and then reanalyzed by HPLC, it was found that all the label had been released from the acyl-enzyme. If this experiment was repeated in the presence of 2 M urea, to maintain the enzyme in a noncatalytically active conformation, 50% of the label was retained. Thus, restoring the acyl-enzyme to native-like conditions resulted in deacylation of the acyl-enzyme, whereas maintaining it under nonactive conditions resulted in a slow loss of the label, presumably due to acid- or base-catalyzed hydrolysis of the exposed ester linkage in the acyl-enzyme. Inactivation of the enzyme by clavulanic acid prior to incubation with the substrate at −40 °C led to no incorporation of the label, as determined by HPLC analysis of the minigel eluant. Similarly, if the substrate was added after the pH was adjusted to 2 at −40 °C, no label was incorporated in the enzyme.

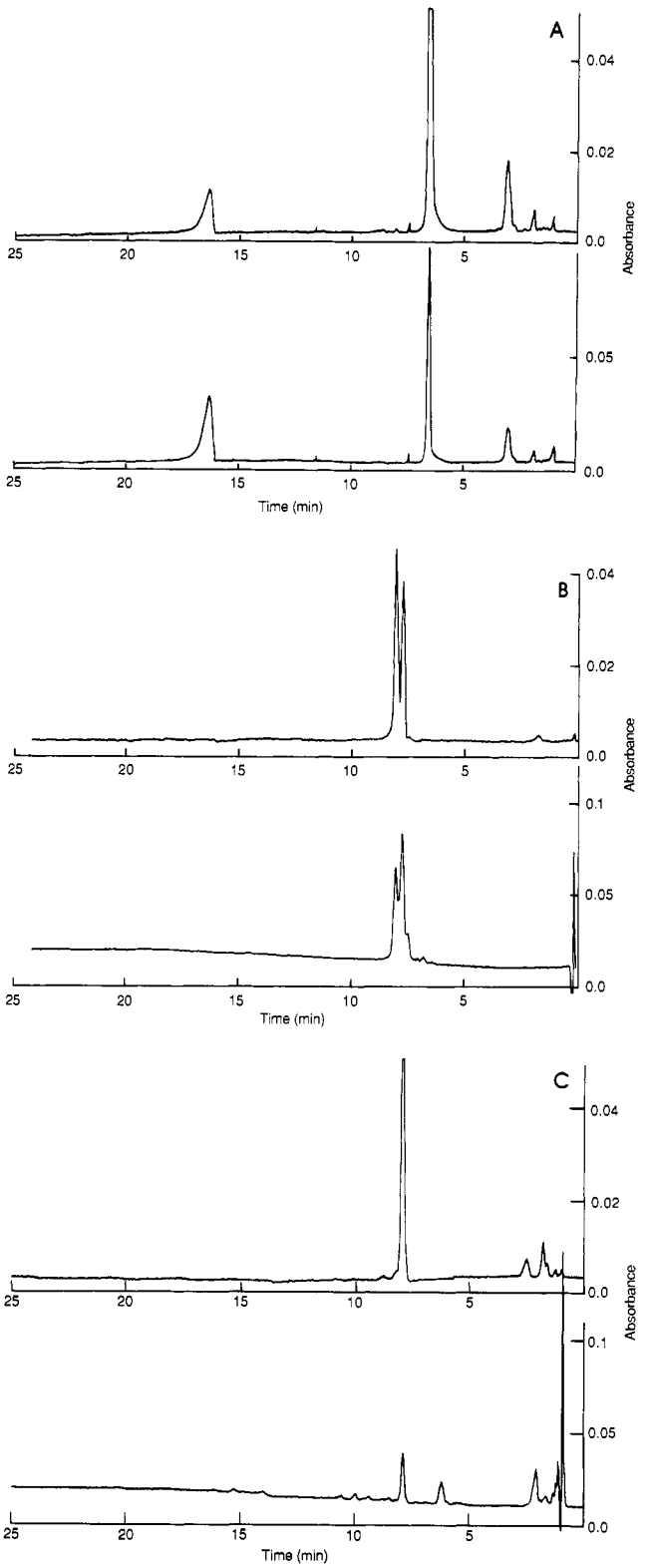

FIGURE 9: HPLC traces of FAP-labeled β-lactamase and peptides. The upper traces are at 305 nm, and the lower are at 280 nm (panel A) and 215 (panels B and C). Panel A shows the results after the initial minigel filtration prior to pepsin digestion. The enzyme elutes at 16.5 min, and the substrate and product elute at 6 and 3 min, respectively. Panel B shows the elution profile after partial pepsin digestion. The labeled peaks had retention times at 7.34 and 8.12 min: the peak at 8.12 min slowly converted to the peak at 7.34 min. Panel C shows the elution profile after trypsin digestion of the labeled fractions from the pepsin digestion. A single labeled peptide with retention time of 8.46 min was observed.

Determination of the Covalently Modified Residue in the Acyl-Enzyme. A sample of the acyl-enzyme from the reaction

with FAP was prepared as outlined above and digested with pepsin at pH 2. Samples were analyzed by HPLC at various times during the digestion. Three labeled peaks were detected at 305 nm. The initial major peak at RT = 17.41 min, corresponding to FAP-labeled β-lactamase, was gradually replaced by two with RT = 7.34 and 8.12 min. Amino acid analysis of the fractions corresponding to the these two peaks indicated that they contained a large peptide consistent with the sequence from residues 41 to 89 of β-lactamase. These fractions were collected from the HPLC, and the pH was adjusted to 7.8. The samples were then digested with trypsin and reanalyzed by HPLC. A single labeled peptide was obtained. Both fractions from the pepsin digestion gave the same labeled peptide on trypsin digestion, with RT = 8.46 min. Amino acid analysis of this peptide indicated that it corresponded to the sequence from Phe-66 to Lys-73. The expected composition is as follows, with the observed composition shown in parentheses: Ala 2 (2), Lys 1 (1), Phe 1 (1), Ser 2 (2), Thr 1 (1), Tyr 1 (≪1). Computer-aided analysis indicates that the F66–K73 peptide is the only region of the amino acid sequence consistent with this amino acid composition. The lack of tyrosine stems from its loss in the sample workup and has also been observed in the corresponding experiments with the enzyme from *B. cereus* (A. K. Tan and A. L. Fink, unpublished results). On the basis of previous reports that various inhibitors modify Ser-70 and that conversion, by site-directed mutagenesis, to other residues except Cys leads to lack of activity, we therefore conclude that the covalent linkage between (furylacryloyl)penicillin and the enzyme involves Ser-70.

Trapping the Acyl-Enzyme at 20 °C. Although FAP is one of the best substrates for the staphylococcal β-lactamase, its turnover number is not very large (k_{cat} = 118 s^{-1} at 25 °C, pH 6.5). We thought it possible, therefore, that the competition between acid quenching and deacylation for the acyl-enzyme might favor the denaturation even at 20 °C. Consequently, we repeated the above acyl-enzyme trapping experiment at 20 °C rather than at −40 °C. After the acid quench the sample was analyzed by HPLC and showed the covalent attachment of the FAP. The stoichiometry was calculated to be 0.8:1. Digestion of the acyl-enzyme with pepsin and trypsin yielded the same labeled peptide (and same amino acid composition) as from the subzero temperature experiment, confirming the same acyl-enzyme intermediate.

The fact that the acyl-enzyme can be trapped in this manner at 20 °C means not only that deacylation must be at least partially rate-limiting but also that the partitioning between deacylation and acid-quenched denaturation must favor the latter. This is in contrast to the case with the enzyme from *B. cereus* in which the acyl-enzyme can be trapped at subzero temperature but not at 20 °C (A. K. Tan and A. L. Fink, unpublished observations). Pratt et al. (1988) have recently reported evidence that benzylpenicillin and dansylpenicillin form acyl-enzyme intermediates in the reaction with β-lactamase from *S. aureus* with rate-limiting deacylation at 0 and 25 °C at pH 7.5 and 9.0. Thus, deacylation appears to be the rate-limiting step for good substrates for this enzyme under "normal" conditions.

Acknowledgments

We are grateful for the assistance of Gabriel Llabres, Sally Swedberg, Martin J. Gartland, June Sanders, and Qi Xiaolin with some of the experiments. We thank Tony Pickard for preparation of the β-lactamase.

Registry No. FAP, 28812-95-5; Benzylpen, 61-33-6; DNPpen, 123994-07-0; DNSpen, 81416-73-1; Ser, 56-45-1; MeOH, 67-56-1; ethylene glycol, 107-21-1; nitrocefin, 41906-86-9; β-lactamase, 9073-60-3.

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Kinetic Mechanism of the Type II Calmodulin-Dependent Protein Kinase: Studies of the Forward and Reverse Reactions and Observation of Apparent Rapid-Equilibrium Ordered Binding†

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Appendix: Various Cases of Rapid-Equilibrium Ordered Bireactant Mechanisms—Their Bases and Differentiation

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Received May 8, 1989; Revised Manuscript Received August 18, 1989

ABSTRACT: The kinetic reaction mechanism of the type II calmodulin-dependent protein kinase was studied by using its constitutively active kinase domain. Lacking regulatory features, the catalytic domain simplified data collection, analysis, and interpretation. To further facilitate this study, a synthetic peptide was used as the kinase substrate. Initial velocity measurements of the forward reaction were consistent with a sequential mechanism. The patterns of product and dead-end inhibition studies best fit an ordered Bi Bi kinetic mechanism with ATP binding first to the enzyme, followed by binding of the peptide substrate. Initial-rate patterns of the reverse reaction of the kinase suggested a rapid-equilibrium mechanism with obligatory ordered binding of ADP prior to the phosphopeptide substrate; however, this apparent rapid-equilibrium ordered mechanism was contrary to the observed inhibition by the phosphopeptide which is not supposed to bind to the kinase in the absence of ADP. Inspection of product inhibition patterns of the phosphopeptide with both ATP and peptide revealed that an ordered Bi Bi mechanism can show initial-rate patterns of a rapid-equilibrium ordered system when a Michaelis constant for phosphopeptide, K_{ip}, is large relative to the concentration of phosphopeptide used. Thus, the results of this study show an ordered Bi Bi mechanism with nucleotide binding first in both directions of the kinase reaction. All the kinetic constants in the forward and reverse directions and the K_{eq} of the kinase reaction are reported herein. To provide theoretical bases and diagnostic aid for mechanisms that can give rise to typical rapid-equilibrium ordered kinetic patterns, a discussion on various sequential cases is presented in the Appendix.

The type II calmodulin-dependent protein kinase (CaM kinase II)[1] is a multifunctional enzyme found most prominently in the brain, where it appears to regulate a diverse number of physiological processes [for review see Nairn et al. (1985), Kennedy et al. (1987), Schulman and Lou, (1989), and Colbran et al. (1989a)]. Many laboratories, including ours, have studied the regulation of CaM kinase II activity by Ca^{2+}/calmodulin and autophosphorylation to elucidate molecular mechanisms by which this enzyme transforms transient Ca^{2+} signals into biochemical responses. Specific regulatory features have been identified: (1) Binding of Ca^{2+}/calmodulin to CaM kinase II removes an autoinhibitory effect which is attributed to an inhibitory region located between the catalytic and calmodulin-binding domains of the protein kinase (Payne et al., 1988; Colbran et al., 1989b). (2) Although calmodulin is not required for nucleotide binding to CaM kinase II, the activator increases the binding affinity (King, 1988; King et al., 1988). (3) Following binding of calmodulin, autophosphorylation of CaM kinase II is required to fully activate

† This work was supported by the National Science Foundation (DMB-8502706 and DMB-8816258).

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[1] Abbreviations: CaM kinase II, type II calmodulin-dependent protein kinase; EDTA, ethylenediaminetetraacetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); P-syntide, phosphorylated syntide.

0006-2960/90/0429-153$02.50/0