Kinetic and Structural Characterization of Reversibly Inactivated β-Lactamase†

Anthony L. Fink,* Kathie M. Behner, and Anthony K. Tan
Department of Chemistry, The University of California, Santa Cruz, California 95064
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ABSTRACT: The reversible inhibition of β-lactamase I from *Bacillus cereus* by cloxacillin, methicillin, and nafcillin has been systematically investigated. For these substrates the enzymatic reaction involves partitioning of the substrate between turnover and inhibition. Typically, concentrations of several hundred millimolar are necessary for complete inactivation. The completely inactivated enzyme could be formed by incubation at temperatures above 20 °C, where inhibition competes more effectively with turnover, and then stabilized by dropping the temperature to 0 °C or lower. The inactivated enzyme was rapidly separated from unreacted substrate and product at low temperature by centrifugal gel filtration or ion exchange and examined by far-UV circular dichroism for evidence of a conformational change. At pH 7 the inactivated enzyme had a secondary structure essentially identical with that of the native enzyme. The fluorescence emission spectrum of the inactivated enzyme (at pH 7) was also identical with that of the native enzyme. However, the inactivated enzyme was found to be considerably more sensitive to thermal denaturation, to acid-induced conformational isomerization, and to trypsinolysis than the native enzyme. We conclude from the circular dichroism results that the structure of the reversibly inactivated enzyme cannot be significantly different from that of the native enzyme. Therefore, previous findings that have been interpreted as indicating a *major* conformational change must be reevaluated. From examination of the low-resolution crystallographic structure of the enzyme we propose that the most likely cause of the inactivation is an alternate conformational state of the acyl-enzyme intermediate involving movement of one or more of the α-helices forming part of the active site. Such a structural effect could leave the secondary structure unchanged but have significant effects on the tertiary structure, catalysis, mobility, and susceptibility to trypsin and denaturation. We propose that the underlying physical reason why certain β-lactam substrates bring about this "substrate-induced deactivation", or suicide inactivation, of the enzyme is due to the presence of the alternative acyl-enzyme conformation of similar free energy to the productive one, in which one (or more) essential catalytic group is no longer optimally oriented for catalyzing deacylation. Thus for substrates with a slow rate of deacylation ($\leq 100\ s^{-1}$) the conformational transition can compete effectively on the time scale of the turnover reaction.

That certain substrates can induce inactivation of β-lactamases has been known for many years. The phenomenon is often called "substrate-induced deactivation", involves the *reversible* inactivation of the enzyme, and has been reviewed in detail by Pain and Virden (1979). Citri et al. (1976) coined the term "A-type substrates" for those β-lactams that bring about this reversible inhibition. A-type substrates typically undergo many thousands of turnovers for each inactivation event. The phenomenon has been observed with several class A β-lactamases. Investigations by Citri, Waley, and Pain and their co-workers have resulted in a number of observations that are consistent with a conformational change occurring with the inactivation. For example, Kiener and Waley (1977) used tritium–hydrogen exchange to show that A-type substrates caused substantial changes in the number and reactivity of the peptide hydrogens in exchange-in and exchange-out experiments. Both chemical cross-linking and antibodies have been used to "lock" the enzyme in either the active or inactive form (Klemes & Citri, 1979; Pain & Virden, 1979; Farrer & Virden, 1980; Carrey et al., 1984).

Sulfate ions have been shown to minimize the effect of A-type substrates (Pain & Virden, 1979; Persaud et al., 1986), also suggesting the involvement of protein structural changes. Virden et al. (1975, 1978; Persaud et al., 1986) found that in quinacillin-inactivated β-lactamase the inhibitor was covalently attached to the enzyme. Kiener et al. (1980) demonstrated that the inactivated enzyme contained 1 equiv of a penicilloyl group and that the inactivated enzyme was more susceptible to denaturation by urea than the native enzyme. They concluded that inactivation involved the formation of a normal intermediate, the acyl-enzyme, which was "prone to unfold". This conclusion has recently been questioned by Persaud et al. (1986).

In view of the unusual nature of this enzyme–substrate interaction and the potential information that an understanding of it might provide in terms of designing new β-lactam antibiotics with resistance to the β-lactamases, we have undertaken to characterize the inactive form of the enzyme in detail. In order to accomplish this, we first carried out a systematic investigation of the reversible inactivation process. We used three typical type A substrates, cloxacillin, methicillin, and nafcillin, whose structures are shown in Chart I. Our findings, in particular the susceptibility of the inactivated enzyme to denaturation by heat and low pH, explain some previously reported contradictory observations. We determined conditions to stabilize the reversibly inactivated enzyme and used circular dichroism and fluorescence to ascertain its conformation. Our results indicate that at pH 7 the secondary structure of the inactivated enzyme is essentially identical with that of the native enzyme.

Experimental Procedures

Materials

β-Lactamase I from *Bacillus cereus* strain 569/H/9 was prepared according to the method of Davies et al. (1974) and stored in 50 mM sodium phosphate buffer, pH 7.0, at 4 °C. The enzyme was homogeneous by sodium dodecyl sulfate

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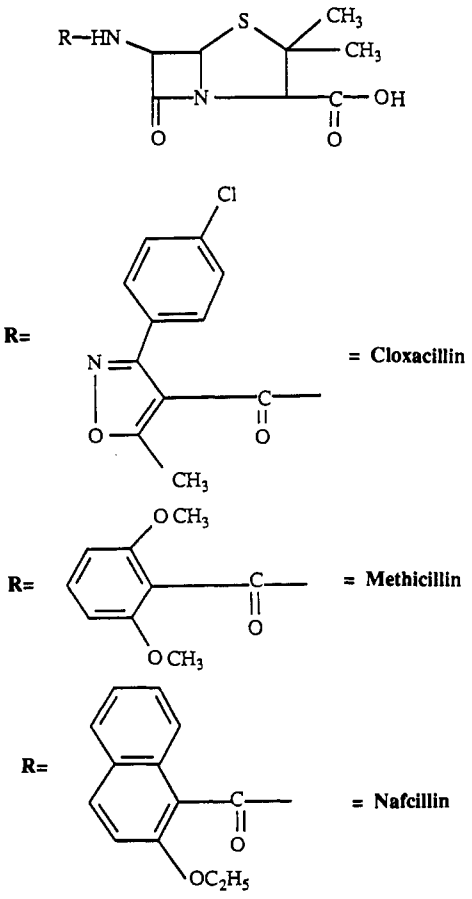
Chart I



(SDS)–polyacrylamide gel electrophoresis. Assays for β-lactamase II (Davies et al., 1974) indicated its absence. Cloxacillin, methicillin, and nafcillin were obtained from Sigma. 6-(β-Furylacryloyl)penicillin acid (FAP)[1] and PADAC were purchased from Calbiochem-Behring. HPLC-grade methanol from Fisher was used in making aqueous–methanol solutions. Other materials were reagent grade or better.

Methods

Inactivation. Standard conditions for inactivation of the enzyme involved incubating 1–4 μM β-lactamase with 250 mM cloxacillin at pH 7.0, 20–22 °C, for 15–30 min. The pH was maintained either by using 0.25 M sodium phosphate buffer or by using 0.05 M sodium phosphate buffer and adding aliquots of 0.2 M NaOH to maintain the pH constant. To measure the rate and amount of inactivation, aliquots were removed from the incubation mixture, diluted 1000-fold in 0.05 M sodium phosphate buffer to reduce the cloxacillin concentration, and then assayed, as outlined below, to measure the amount of inactivation and the rate and amount of reactivation. The rate of inactivation was determined from the time-dependent decrease in activity. Control incubations were run simultaneously in which the enzyme was incubated under the same conditions but in the absence of inhibitor.

The rate of cloxacillin hydrolysis during the incubation was determined by following the formation of product and loss of substrate by HPLC. Solutions of 125 and 250 mM cloxacillin were incubated separately with 3.9 μM β-lactamase. Aliquots of the incubations were removed at intervals and diluted 100- or 200-fold prior to injection. Separation of cloxacillin and its hydrolyzed product was achieved on a C18 reverse-phase column as indicated below.

The inactivation was also followed directly by spectrophotometric monitoring of the rate of substrate hydrolysis. Cuvettes of 0.1-mm path length were used because of the high background absorbance. The reactions were followed at the lowest wavelength where the initial absorbance was below 2 for the concentration studied. Typical wavelengths were 305 nm for methicillin, 265 nm for cloxacillin, and 340 nm for nafcillin.

Reactivation Assay. The amount of inactive enzyme initially present, the rate of reactivation, and the amount of reactivation were determined by assaying with a good substrate, usually (furylacryloyl)penicillin or PADAC. Benzylpenicillin was occasionally used. The standard FAP assay system consisted of 2–3 mM (furylacryloyl)penicillin (K_m = 3 × 10^{-4} M) in 0.05 M sodium phosphate buffer at pH 7.0 and 21 °C. The reaction was monitored at 345 nm ($\Delta\epsilon$ = 850 M^{-1} cm^{-1}) with a Cary 118 or 219 spectrophotometer. The standard PADAC assay consisted of 36–50 μM PADAC (K_m = 7.7 × 10^{-6} M) in 0.05 M sodium phosphate buffer, pH 7.0, at 21 °C. The reaction was monitored at 573 nm ($\Delta\epsilon$ = 16050 M^{-1} cm^{-1}).

The initial amount of inactive enzyme and the rate and amount of reaction were determined by computer analysis using the program REDUCE, developed in this laboratory in collaboration with Dr. S. J. Koerber. The algorithm used assumed that the reactivation was a first-order process; thus the steady-state kinetics are represented by

$$v = k_{cat}[E_0](1 - e^{-k_{react}t})[S]/(K_m + [S]) \quad (1)$$

Data for the reactivation kinetics were fit to the expression:

$$v = \frac{[A + B(1 - e^{-kt})][S]}{K_m + [S]}$$

where $A/(A + B)$ is the fraction of active enzyme at the beginning of the assay and $A + B$ is equivalent to V_{max}. The amount of reactivation was measured by comparing the maximum velocity of the enzyme incubated in the absence of inhibitor vs. that of the enzyme incubated with inhibitor. Typical reactivation assay curves are shown in Figure 1. Reactivation of purified inactivated enzyme was also followed by HPLC. After inactivation under standard conditions the inactivated enzyme was separated from excess substrate and products by minigel chromatography (see below) at 0 °C. The sample was allowed to reactivate at 0 °C, and aliquots were injected into the HPLC at periodic intervals to measure the rate of increase in cloxacillin penicilloate.

Effect of pH on Inactivation. Incubation of 0.25 μM β-lactamase and 250 mM cloxacillin was carried out in buffers of pH 4–10. Sodium acetate was used for pH 4 and 5, sodium phosphate for pH 6, 7, and 8, and sodium pyrophosphate for pH 9 and 10. The buffer concentrations were 0.5 M to prevent pH changes caused by cloxacillin hydrolysis. Aliquots were removed as a function of time and assayed with the standard reactivation assay to measure the amount of inactivated enzyme.

Effect of pH on Reactivation. β-Lactamase was inactivated by the standard incubation. Aliquots were assayed by either PADAC or FAP according to the standard reactivation assay except that the pH was varied from 4.0 to 10.0, by use of sodium acetate, sodium phosphate, or sodium pyrophosphate buffers. At pH 8.5 and above, control experiments were run

[1] Abbreviations: FAP, (furylacryloyl)penicillin; PADAC, [2-[[*p*-(dimethylamino)phenyl]azo]pyridinio]cephalosporin; CD, circular dichroism; pH*, apparent protonic activity, i.e., apparent pH of aqueous–methanol solvent system, as measured with a glass pH electrode; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

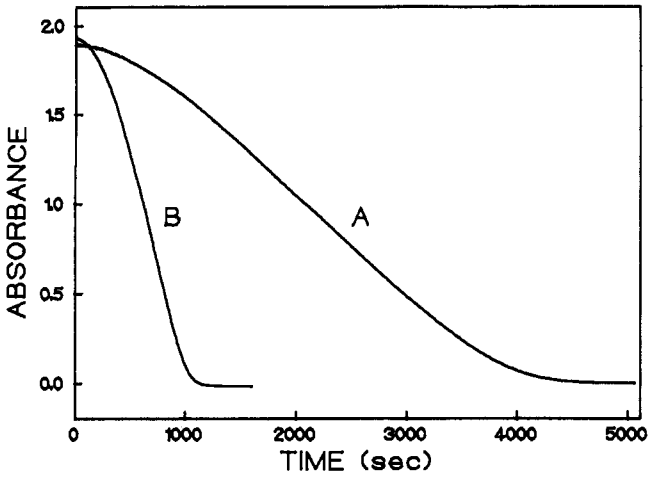


FIGURE 1: Reactivation assay curves. The conditions were pH 7.0 and 21.0 °C, and the substrate was PADAC (35 μM). β-Lactamase (4.8 μM) was inactivated with cloxacillin (250 mM) and diluted 100- (B) and 1000-fold (A) into the PADAC assay mixture. The initial horizontal slope, seen most clearly in curve A, represents inactivated enzyme. The subsequent exponential decrease in absorbance reflects reactivation. The lower part (later times) of the curve represents the last phase, substrate depletion, in the catalytic hydrolysis of PADAC. The rate constant for reactivation under these conditions was 1.3×10^{-3} s^{-1}. The reactivation appears much faster in curve B because the [active enzyme]/[PADAC] ratio is much higher than in curve A, and the substrate is exhausted prior to complete reactivation (see text).

to determine the rate of spontaneous hydrolysis of the substrate in the reactivation assay.

HPLC Separations. A Beckman gradient HPLC system equipped with a Model 165 detector and C18 reverse-phase column was used to analyze substrate and product solutions. The column was 25 × 0.46 cm, with 10-μm microporous silica-based packing. Solvent A was 10 mM sodium phosphate buffer, pH 3. Solvent B was acetonitrile. The gradient began at 20% B; after 1 min the concentration of B was increased to 42% over 2 min. After 1 min solvent B was raised to 90% over 5 min. The flow rate was then increased from 1 to 1.5 mL/min and the gradient held constant for 4 min. The composition was then changed to 20% solvent B over a 15-min period.

For the separation of proteins and peptides a silica-based reverse-phase C3 ultrapore RPSC (Beckman) column, 5-μm, extensively end-capped 7.5 × 0.46 cm column was used. Solvent A was water containing TFA (0.1%). Solvent B was acetonitrile containing TFA (0.1%). The gradient began at 5% solvent B. After 2 min a linear ramp (over 15 min) to 45% solvent B was started. After a further 8 min the gradient was decreased to 5% solvent B over 15 min. Flow rate was 1 mL/min.

Fluorescence of Inactivated Enzyme. β-Lactamase (4 μM) was incubated in the standard fashion and the inhibited enzyme purified by centrifugal chromatography (see below). The fluorescence emission spectrum of the eluent (1 mL) was obtained by excitation at 285 nm at 2 °C. The excitation and emission maxima were 285 and 336.5 nm, respectively.

Chromatographic Separation of Inactivated Enzyme. Excess substrate or product was removed from the inactivated enzyme by using centrifugal minigel chromatography. This procedure permits very rapid separations and was necessitated by the short lifetime of the inactivated enzyme. Both Sephadex G-25 (superfine grade) and DEAE-Sephadex were found to be suitable supports. The Sephadex G-25 was prepared by equilibration with 50 mM sodium phosphate buffer, pH 7; the DEAE-Sephadex was preequilibrated with 50 mM Tris buffer, pH 7. A 6-mL disposable syringe, containing a Millipore Teflon (0.5-μm) filter to support the packing, was filled with the slurry. The syringe was then spun for 15 min in a Jouan Model GR411 centrifuge to pack the support and remove excess liquid. The packed-bed height was 4 cm. Sample, 1 mL, was applied to the top of the temperature-equilibrated column, which was then spun at 4000 rpm for 2 min. Temperatures of 0 °C or below were used in these experiments in order to minimize the amount of reactivation occurring during the separation. The eluent was collected in a small conical plastic vial located between the bottom of the column and the bottom of the centrifuge tube.

Circular Dichroism Experiments. Circular dichroism spectra were collected with a modified, computer-controlled Jasco Model J-20 instrument. Data were collected at 1-nm intervals with time averaging. A thermostated sample cell holder with dry nitrogen gas purging was used for low-temperature spectra. The following procedure was used to obtain the spectra of the inactivated enzyme at pH 7.0. Cloxacillin (250 mM) and β-lactamase (8.1 μM) were incubated in 1 mL of 50 mM buffer at 22 °C, for 10 min. As the incubation progressed, 70 μL of 0.5 M NaOH was added to maintain the pH at 7.0. The solution was put on ice to lower the temperature to 0 °C. Then 0.5 mL of cold methanol was added to the enzyme–cloxacillin mixture to make the solution 30% (v/v) methanol. The solution was immediately pipetted onto a pH 7, DEAE-Sephadex column prepared as above and centrifuged for 2 min at −10 °C. The eluent was then transferred to a precooled 0.5-mm path length cell in the spectropolarimeter, and the circular dichroism spectrum was measured at −10 °C between 240 and 195 nm at frequent intervals over a 5-h period. Simultaneously, an aliquot was assayed in the standard reactivation assay at −10 °C in 30% methanol.

In a related experiment, after isolating the inactivated enzyme at pH 7, as above, the pH was then changed to pH 4.5 and the CD spectrum obtained prior to returning the pH to 7. In this experiment, after centrifugation the eluent was combined with 10 μL of 1.5 M HCl to drop the pH to 4.5. An aliquot of the pH 4.5 solution was diluted 1000-fold and assayed at −10 °C with FAP (pH 4.5 in 50 mM sodium acetate–30% methanol). The remainder of the solution was pipetted into the precooled CD cell at −10 °C and scanned between 250 and 195 nm. The solution was then restored to pH 7 by the addition of NaOH and rescanned between the same wavelengths. The solution was stored at 5 °C for 3 days and scanned a third time under the same conditions. As a control the native enzyme was scanned in 30% methanol, at pH 7 and 4.5 at −10 °C.

Kinetics experiments were routinely carried out in duplicate or triplicate. Kinetic simulations were done with the program KINSIM (Barshop et al., 1983) or an analogous program running on a microcomputer.

Results and Discussion

Our approach was first to determine the conditions necessary to maximize the yield and stability of the inactivated enzyme. This would then facilitate its subsequent isolation and structural characterization. The investigation thus consists of three main parts: studies of (1) the inactivation reaction, (2) the reactivation process, and (3) the inactivated enzyme itself. The terms inactivated and inhibited are used interchangeably. Unless otherwise stated, the data refer to cloxacillin as the inhibitor.

A number of previous investigations support the hypothesis that the β-lactamase catalytic reaction pathway involves an

acyl-enzyme intermediate in which a transient covalent bond is formed between the β-lactam carbonyl and Ser-70 of the enzyme (Fink, 1985). Kiener et al. (1980) and Persaud et al. (1986) have shown that the properties of the reversibly inactivated form of the enzyme are consistent with those of an inactive form of the acyl-enzyme intermediate.

Reactivation Assay. The procedure chosen to determine the initial amount of inactive enzyme, the rate of reactivation, and the amount of reaction was to add a diluted aliquot containing the inactivated enzyme to a solution of a good substrate, benzylpenicillin, PADAC, or FAP, under substrate-saturating conditions (i.e., $[S_0] \gg K_m$). Experiments were undertaken to determine the maximum amount of type A substrate that could be present in the assay mixture without having any detectable effect. These showed that concentrations of cloxacillin of 2.0 mM or less had no effect on the rate of β-lactamase-catalyzed hydrolysis of benzylpenicillin or PADAC. This observation also means that the binding affinity of cloxacillin, acting as a competitive inhibitor, must be greater than 2.0 mM. In the case of nafcillin the maximum concentration that could be present without competitive inhibition effects was 0.01 mM.

It is important in experiments of the sort carried out in this study that sufficiently high substrate and low enzyme concentrations be used in the assays for reactivation. There must be sufficient substrate present so that when the reactivation is complete, there is still substrate present. If these conditions are not met, erroneous values of the rate and amount of reactivation may be obtained. The critical factor is the ratio of substrate concentration to active enzyme. If insufficient substrate is present, the observed reactivation rate will appear faster, and the amount of reactivation smaller, than the real values (Figure 1). The observed shape of the reactivation curve appears "normal" (Figure 1B) even when insufficient substrate is present, due to the compensating absorbance changes due to decreasing substrate ($[S] < K_m$) and increasing concentration of active enzyme from reactivation. Plots of the observed rate of reactivation against the reciprocal of the dilution factor or enzyme concentration may be used in such cases to extrapolate to the "true" value of k_{react} or to demonstrate that a suitable [S]/[E] ratio is present in the assay.

The effect of length of incubation time with cloxacillin on the inactivation and subsequent reactivation was investigated. Enzyme was inactivated in the standard manner, and after complete inactivation (5 min) aliquots were assayed at frequent time intervals over a 2-h period. No changes in the amount of initially inactive or finally reactivated enzyme concentration, nor in the rate of reactivation, were observed. These observations only hold, however, for inactivation incubations and reactivation assays at pH 7 and for temperatures below 25 °C. When β-lactamase was inactivated under standard conditions and assayed at temperatures below 25 °C, complete reactivation occurred. However, if the assay temperature was 30 °C or higher, significantly lower reactivation was found. This was due to the greater sensitivity of the inactivated enzyme to thermal denaturation compared to the native enzyme, for which the thermal denaturation transition begins around 40 °C at pH 7. Similarly, at pH <5 the inactivated enzyme was very susceptible to acid-induced denaturation.

Complete recovery of enzymatic activity was observed with all three A-type substrates when the incubation mixtures were allowed to react for sufficient time periods at 20–25 °C. The time period was much shorter for methicillin and nafcillin, due to their faster rate of reactivation.

Table I: Kinetic Characterization of Inactivation and Reactivation of β-Lactamase by Cloxacillin, Methicillin, and Nafcillin

inhibitor	k_{inact}[a] (s^{-1})	k_{react}[b] (s^{-1})	max inact[c] (%)	K_d[d] (mM)
cloxacillin	6.5×10^{-3}	4.0×10^{-3}	98	16.0
nafcillin	1.6×10^{-1}	1.3×10^{-2}	100	7.1
methicillin	1.5×10^{-3}	1.2×10^{-2}	82	15.4

[a] The rate of inactivation measured by direct observation at 21 °C, pH 7.0; see legend to Figure 2 for further details. [b] The rate of reactivation at 25 °C, pH 7.0. Assayed with PADAC (cloxacillin) or benzylpenicillin (methicillin and nafcillin). [c] The maximum amount of inactivation at saturating inhibitor concentration, extrapolated from the data of Figure 3. [d] K_d is defined as the concentration of inhibitor for which half the maximum inactivation was observed. The values were obtained from curve fitting a hyperbola to the experimental data. See also Figure 3.

Direct Observation of Inactivation. The hydrolysis of cloxacillin, nafcillin, and methicillin is accompanied by small changes in the near-UV region of the absorbance spectrum. By using very short path length cells, to minimize the background absorbance due to the high concentration of the β-lactam, it was possible to directly observe the decrease in rate of substrate hydrolysis as inactivation occurred (Figure 2). Rates of inactivation obtained from these experiments were in good agreement with those obtained by the above assay method (Table I).

Effect of Inhibitor Concentration on Amount of Inactivation. Previous reports regarding type A substrates have suggested that the amount of inactivation occurring was a function solely of the combination the particular β-lactamase and inhibitor used. Our observations show that if sufficiently high inhibitor concentration is used then close to complete inactivation will occur. This is illustrated for cloxacillin, methicillin, and nafcillin in Figure 3. The amount of inactivated enzyme is a function of the inhibitor concentration. The data fit saturation binding curves, with the K_d values shown in Table I. The value of K_d is a complex expression of K_s (the inhibitor dissociation constant), the ratio of reactivation to inactivation, and the ratio of acylation to deacylation. For nafcillin, but not cloxacillin, the value of K_d is orders of magnitude greater than K_i (the inhibition constant as a competitive inhibitor). Note that very large concentrations of inhibitor may be required for complete inactivation (hundreds of millimolar), much higher than those used in most previous investigations.

The rate of cloxacillin hydrolysis during incubation was monitored by HPLC, using 125 mM and 250 mM cloxacillin. As anticipated, these experiments showed a faster rate of cloxacillin hydrolysis when less inhibitor was present. After an initial rapid rate of product formation (during the inactivation process) a slow rate of product formation was observed due to hydrolysis by the small amount of noninhibited enzyme. Extrapolation to zero time allowed an estimate of the turnover/inactivation ratio, which was 3.8×10^3 for cloxacillin at pH 7.0, 21 °C. This is in good agreement with the value estimated from the reported value of k_{cat} (Citri et al., 1976) and the rate of inactivation, namely, 1.8×10^3.

In order to determine whether the hydrolyzed product of cloxacillin had any inhibitory effect, cloxacillin was hydrolyzed at pH 11; the reaction was monitored by HPLC. At 250 mM, under the standard incubation conditions, the penicilloic acid product had no effect on the enzyme when assayed with the reactivation assay.

Inactivation by Methicillin and Nafcillin. Typical kinetic data for these substrates are given in Table I, which shows the rates of inactivation and reactivation, the maximum amount of inactivation, and the estimated K_d. We observed,

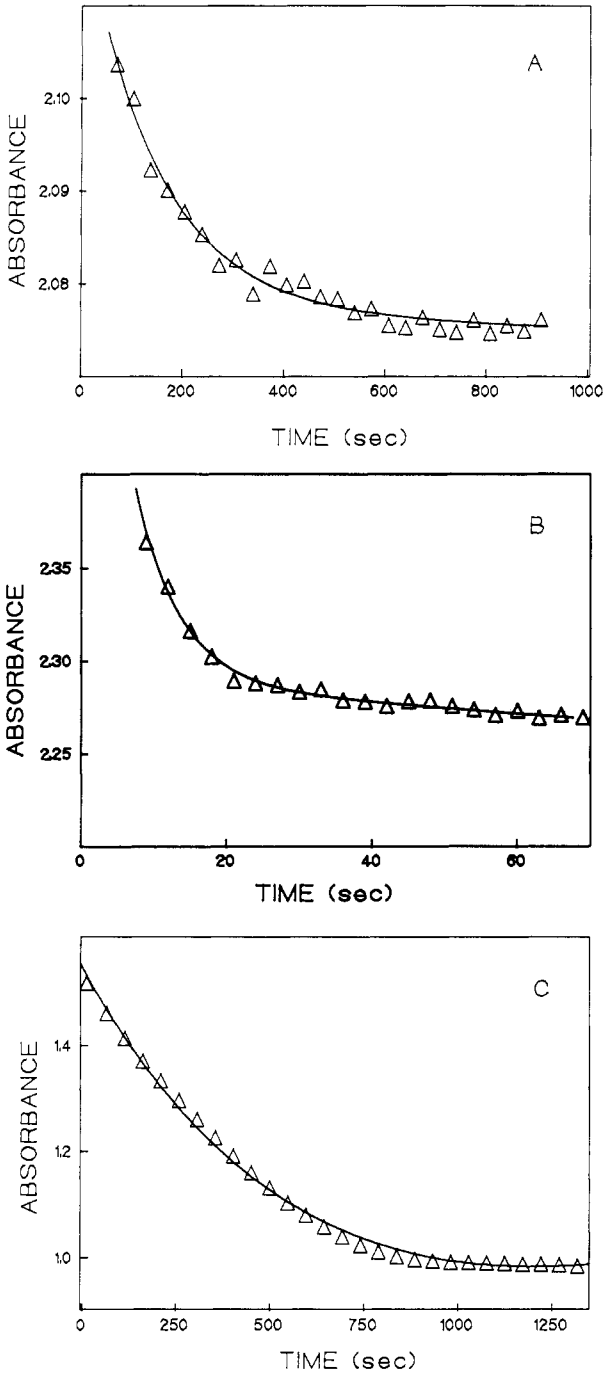


FIGURE 2: Direct observation of penicillin-induced inactivation. In each case, the starting concentration of the penicillin is at least $100K_m$. For each inhibitor the reaction appears as a first-order (monoexponential) decrease in absorbance followed by a zero-order reaction. The former corresponds to inactivation and the latter to the very limited amount of turnover occurring due to less than 100% inactivation. Panel A shows the effect of cloxacillin (200 mM) on β-lactamase (4 μM) monitored at 265 nm. Panel B shows the results for nafcillin (100 mM) with 1 μM β-lactamase monitored at 340 nm. Panel C shows the data for methicillin (400 mM) with 4 μM β-lactamase at 305 nm. The experiments were at 21 °C, pH 7.0.

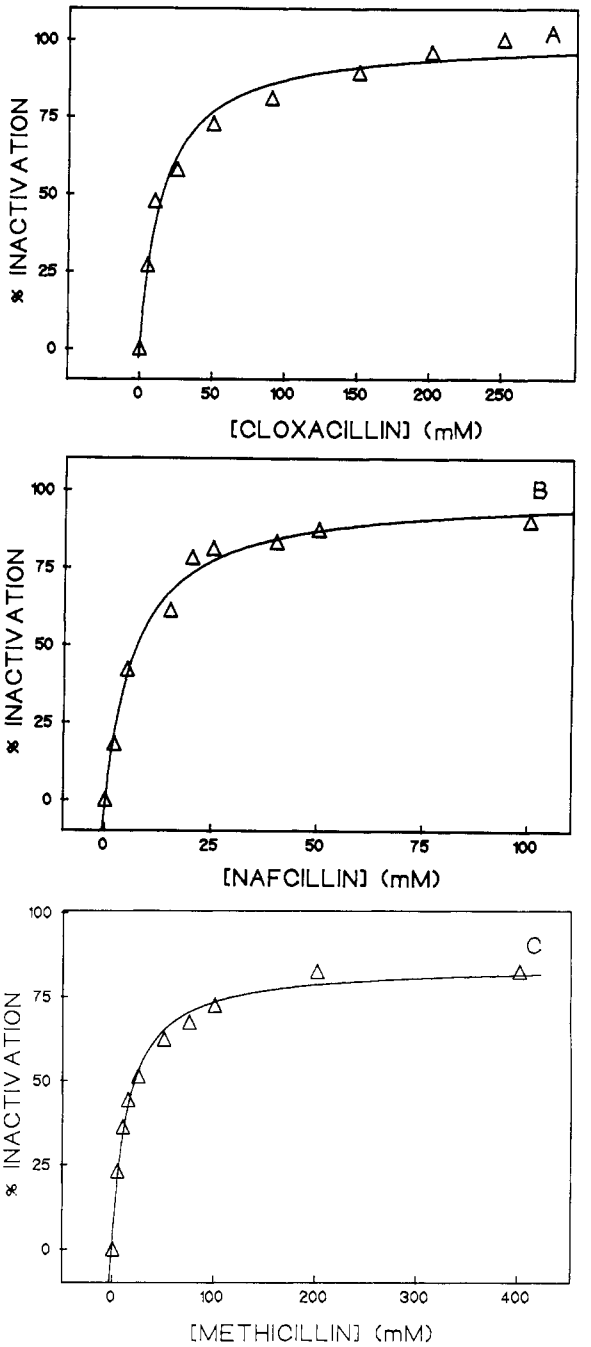


FIGURE 3: Effect of inhibitor concentration on amount of inactivation. The inactivation was done at pH 7.0, 25 °C, with 1 μM enzyme. (A) Cloxacillin; (B) nafcillin; (C) methicillin.

especially with methicillin because of the combination of relatively fast reactivation rate and fast turnover rate, that relatively rapid regeneration of active enzyme occurred in the inactivation incubations. Thus the maximum concentration of inactivated enzyme is present for only a relatively short time period. Citri et al. (1976) report values of k_{cat} (30 °C, pH 7) for cloxacillin, methicillin, and nafcillin to be 53, 130, and 60 s^{-1}, respectively.

Effect of pH on Inactivation. The rate of inactivation is shown as a function of pH in Table II. With 250 mM cloxacillin the enzyme was completely inactivated at all pH values in a period of 5 min or less at 25 °C. The rate of inactivation increases with increasing pH above pH 7, and with decreasing pH below pH 6. At pH 4.5 and 4 the rate of inactivation was so rapid it could not be measured. This sharp increase in the rate of inactivation was observed for incubation at 3 °C as well. As discussed later, little or no *reactivation* occurs on incubation at pH ≤4.5. The control enzyme solution (incubated with no cloxacillin), however, is stable under these conditions.

Both *B. cereus* and *Staphylococcus aureus* β-lactamases have been reported to undergo an acid-induced, at least partially reversible inactivation (Davies et al., 1974; Pain & Virden, 1979). We have found that for the enzyme from *B.*

Table II: Effect of pH on Rate of Cloxacillin-Induced Inactivation and Reactivation of β-Lactamase

pH	k_{inact}[a] (s^{-1})	k_{react}[b] (s^{-1})
4.0	≫0.25	
4.5	>0.25	0
5.0	4.8×10^{-2}	4.3×10^{-3}
6.0	1.2×10^{-2}	3.8×10^{-3}
7.0	1.2×10^{-2}	3.0×10^{-3}
8.0	3.6×10^{-2}	3.2×10^{-3}
8.5	4.9×10^{-2}	4.2×10^{-3}
9.0	4.9×10^{-2}	4.8×10^{-3}
9.5		6.9×10^{-3}
10.0	7.7×10^{-2}	4.2×10^{-3}

[a] Conditions were 21.0 °C, [E] = 0.25 μM, [cloxacillin] = 250 mM, and 0.5 M sodium phosphate buffer. Assayed with 1.5 mM FAP. [b] β-Lactamase (4.0 μM) was inactivated with 250 mM cloxacillin, pH 7.0, 22 °C. Assayed for reactivation with PADAC (pH 4–8) or FAP (pH 8–10) at 22 °C.

cereus this reaction is fully reversible at temperatures below 20 °C, with pH values as low as 2. The rapid inactivation, and more especially, the lack of reactivation, at pH ≤4.5 suggests that acid-induced *irreversible* denaturation of the inactivated enzyme occurred under these conditions. Previous investigation had shown that the acid-induced denaturation, as monitored by fluorescence, was sensitive to ionic strength, the midpoint of the transition shifting from pH 3.0 to 4.0 on going from 0.05 to 0.5 M NaCl at 22 °C (A. L. Fink and D. Joy, unpublished results). Incubations of β-lactamase with cloxacillin at pH 4.0, followed by assay at pH 7, indicated that some reactivation occurred if the reaction was carried out at 0 °C, whereas none occurred when the incubation was at 20 °C.

The pH dependence of catalysis shows p*K*'s of 5.5 and 8.4 at 25 °C for the free enzyme (k_{cat}/K_m) and p*K*'s in the vicinity of 4 and 9 for the ES complex (k_{cat}) (Hardy & Kirsch, 1984). Thus in catalysis the maximal rate of hydrolysis is found in the neutral pH region, the exact opposite of the case for inactivation. It is possible that the same ionizing groups are involved in both catalysis and inactivation. However, it is also possible that the pH dependence of inactivation reflects the decreased turnover rates at high and low pH and hence apparently more favorable partitioning toward inactivation under conditions where the catalytic rate is slower. This would be the case if the inactivation process were relatively independent of pH, which we believe to be the correct explanation.

Effect of Temperature on Inactivation. β-Lactamase was inactivated by incubation with 250 mM cloxacillin, pH 7.0, at various temperatures over the 1–30 °C range. Higher temperatures were not used in order to preclude thermal denaturation. Aliquots were removed periodically and assayed with the standard PADAC assay for reactivation. The data are shown in the form of an Arrhenius plot in Figure 4. An energy of activation of 23.0 kcal mol^{-1} was calculated for the inactivation process. This large dependence of inactivation rate on temperature means that at lower temperatures the ratio of turnover to inhibition increases significantly. The energy of activation for catalysis is around 10 kcal mol^{-1} (A. L. Fink and S. J. Cartwright, unpublished observations).

Effect of Temperature on Rate of Reactivation. The effect of temperature on the reactivation process was determined by inactivating the enzyme under standard conditions and then assaying with PADAC or FAP at various temperatures, at pH 7.0. The data are shown as an Arrhenius plot in Figure 4; an energy of activation of 12.7 kcal mol^{-1} was calculated.

The data in Figure 4 indicate that the enzyme can be readily inactivated at pH 7, 20 °C, and the resulting inactivated enzyme then stabilized for a significant period by dropping

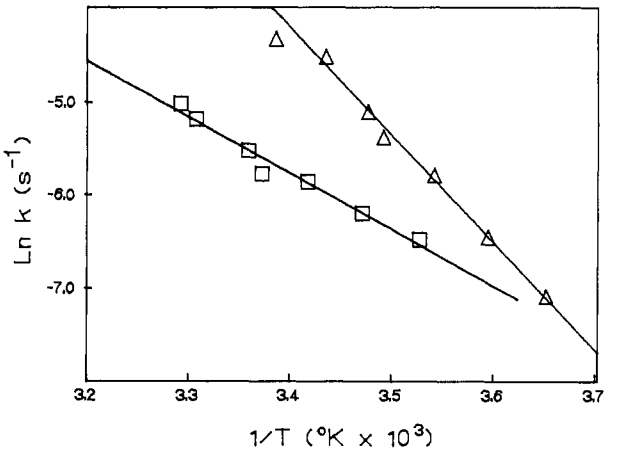


FIGURE 4: Effect of temperature on rates of inactivation and reactivation. The arrhenius plots for inhibition with cloxacillin are shown. The inactivation was done with 250 mM cloxacillin and 0.3 μM β-lactamase, at pH 7.0 (△). Reactivation was at pH 7.0 with PADAC, for 1.25 μM β-lactamase inactivated with 250 mM cloxacillin at 22.0 °C (□).

the temperature to 0 °C or below. The high activation energy for inactivation suggests that the rate-limiting step is not a chemical process but perhaps one involving a structural change in the protein.

Effect of pH on Reactivation. The enzyme was inactivated by the standard incubation procedure, at 22 °C. Aliquots were then assayed with PADAC or FAP at various values of pH, at 25 °C. As shown in Table II, the rate of reactivation was relatively independent of pH with slight increases at both pH extrema. At pH 5 the *amount* of reactivation decreased, and at pH 4.5, 25 °C, there was *no* reactivation, presumably due to irreversible acid-induced denaturation.

Effect of Hydroxylamine on Reactivation. If the rate-limiting step in reactivation involves hydrolysis of the acyl-enzyme, then the rate of reactivation might increase in the presence of the strongly nucleophilic hydroxylamine. Enzyme, inactivated by cloxacillin in the standard fashion, was assayed for reactivation in the presence of 5–50 mM hydroxylamine. Accurate determination of the reactivation rates was complicated by the relatively rapid nonenzymatic hydrolysis of the substrate (due to hydroxylaminolysis); however, it was clear that within experimental error the presence of hydroxylamine caused no increase in the reactivation rate. Thus either the rate-limiting step in reactivation is not hydrolysis of the acyl-enzyme or the acyl-enzyme ester bond is not accessible to hydroxylamine.

Effects of Salts. Salts have long been known to exert stabilizing or destabilizing influences (chaotropic effects) on proteins. Particularly germane to the present study is the report that ammonium sulfate greatly stabilizes the native conformation of β-lactamase from *S. aureus* (Pain & Virden, 1979). In order to determine whether the lifetime of the inactivated enzyme might be affected by the presence of high concentrations of certain salts, we examined the effect of varying the nature and concentration of the salt in the reactivation assay. Ammonium sulfate to 0.5 M had no effect on the assay rate for *native* enzyme. The rate of reactivation of cloxacillin-inactivated enzyme showed no dependence of ammonium sulfate concentration. No change in the amount of reactivation was observed either. 2-(*N*-Morpholino)ethanesulfonic acid (MES) (0.5 M) caused a 32% decrease in the catalytic rate for the control enzyme, and the reactivation rate for cloxacillin-inactivated enzyme showed a parallel decrease. Varying the concentration of sodium phosphate buffer from

50 mM to 0.5 M had no effect on the reactivation rate. Thus the reactivation process is unaffected by salts. This is in contrast to the inactivation reaction which in the case of quinacillin and β-lactamase from *S. aureus* has been shown to be greatly retarded by the presence of high sulfate concentration (Persaud et al., 1986).

Comparison of Reactivation Rates for Acid-Inactivated and Cloxacillin-Inactivated Enzyme. The rates of reactivation were determined at 15 °C, pH 7.0, for enzyme inactivated by exposure to pH 2.0 or incubation with cloxacillin. The pH 2 inactivated enzyme reactivated some 6-fold faster than the cloxacillin-inactivated enzyme (14.5×10^{-3} vs. 2.5×10^{-3} s^{-1}). Consequently, the cloxacillin-inactivated enzyme is likely to be inactive for different reasons than the acid-induced conformation. This was confirmed by the CD experiments discussed subsequently.

Effect of D_2O on Reactivation. To determine whether hydrolysis of the acyl-enzyme bond was involved in the rate-limiting step in reactivation, we investigated the effect of reactivating the enzyme in D_2O, rather than water. The enzyme was inactivated under standard conditions and then assayed in the standard reactivation assay with benzylpenicillin or FAP, as a control, or in a similar assay mixture made up with D_2O. The control experiment with noninhibited enzyme showed a kinetic solvent isotope effect for $k_H/k_D = 1.8$ for V_{max}, consistent with (partial) rate-limiting proton transfer. At pH = pD = 7.0, 21 °C, the reactivation rate for the inactivated enzyme was the same in water and D_2O, $k_H/k_D = 1.00 \pm 0.03$. We also compared the initial velocity of cloxacillin hydrolysis in water and D_2O by direct observation of the reaction of β-lactamase with cloxacillin at 265 nm (see above). At pH = pD = 7.0, 22 °C, we calculated a ratio of $k_H/k_D = 1.9$. A low temperature, high enzyme concentration (0.4 μM), and low substrate concentration (10 mM) were chosen in these experiments to minimize the amount of inhibition.

These observations indicate that proton transfer is involved in the rate-limiting step(s) of cloxacillin hydrolysis, but apparently not in reactivation of the inactivated enzyme. The results are thus consistent with rate-limiting deacylation for cloxacillin hydrolysis and a rate-limiting protein structural change in reactivation.

Effects of Methanol and Low Temperatures on Reactivation Rate. To find conditions for which the inactivated enzyme would be stable for an extended period, we investigated the possibility of using subzero temperatures. Previous investigations (S. J. Cartwright and A. L. Fink, unpublished results) had shown that methanol-based cryosolvents had no adverse effects on the catalytic and structural properties of the enzyme. In addition, the circular dichroism spectrum of the enzyme at pH 7 is unaffected by the presence of methanol below the thermal denaturation transition. The reactivation process was studied at pH * 7.0 at several temperatures between 20 and −15 °C in the presence of 30% (v/v) methanol. Inactivated enzyme was prepared by the standard incubation procedure. Dilutions of 1000-fold were assayed with FAP (2–3 mM); control reactions without the methanol were run at above zero degree temperatures. There was no difference in the amount of reactivation (100%) in the presence or absence of the methanol. The value of k_{cat} for FAP decreased by 60% in the presence of the methanol under the assay conditions (pH* 7.0, 24.2 °C). A similar rate reduction was observed at 10 °C, pH* 7.0, using benzylpenicillin as substrate. The rate of reactivation was not affected by the presence of 30% methanol at 10 °C, pH* 7.0. This observation, along with the lack of effect of hydroxylamine on the rate of reactivation, suggests that competition between water and added nucleophiles is not involved in the reactivation process and that if the rate-limiting step in reactivation is a conformational change, the presence of the methanol does not affect it.

In order to attempt further stabilization of the inactivated enzyme, the combination of low temperature, methanol, and low pH on the reactivation rate was investigated. When the enzyme was inactivated at pH 4 and then assayed at pH 4, no reactivation occurred, presumably due to acid-induced denaturation. In order to probe this phenomenon in greater detail, the following experiment was carried out. After incubation under standard conditions to inactivate the enzyme at pH 7, aliquots (10 μL) were diluted into 90 μL of 30% methanol buffer, either pH* 3, pH* 4, or pH* 5, and incubated for 5 min at 0 °C. Aliquots of these mixtures were then diluted 100-fold into pH* 7, 30% methanol buffer at 0 °C and incubated for 1 h at 0 °C. When these samples were assayed for reactivation, essentially 100% reactivation was observed. We conclude, therefore, that the inactive enzyme is stable at pH* as low as 3, in 30% methanol and 0 °C for *short* periods of time. This suggested that it should be possible to separate the inactivated enzyme from substrate and product by rapid gel filtration or ion-exchange chromatography at pH* 4, 0 °C.

Separation of Inactivated Enzyme from Excess Substrate and Product. The relative short lifetime of the inactivated enzyme, in conjunction with the very large concentrations of inhibitor required to bring about complete inactivation, posed a potential technical problem as far as separating the inactivated enzyme from unreacted substrate and product. We investigated two procedures: gel filtration with Sephadex G-25 and ion-exchange chromatography with DEAE-Sephadex. The basis of the anion-exchange column is the high p*I* (>9) of β-lactamase and the negative charge on the β-lactam. In order to minimize the length of time involved in the separation, we used small columns that could be centrifuged at low temperature.

Both support materials proved satisfactory. For DEAE-Sephadex yields of 90–100% (2–4-cm bed height) native β-lactamase and 40–50% inactivated enzyme were determined by using 1 mL of sample and 2-min centrifugation at 4000 rpm. Under these conditions application of a 1-mL sample of 250 mM cloxacillin to the column resulted in a concentration of 3 μM cloxacillin in the eluent. With Sephadex G-25 similar recoveries of enzyme, and the slightly higher of cloxacillin (10 μM), were obtained in the eluent. The replacement of the pH 7 buffer in the above experiments with 30% methanol, pH* 4 or 7, had little effect on the yields. As noted above, the *inactivated* enzyme is recovered in much poorer yields from these columns. This is another example of the changed properties of the inhibited enzyme compared to the native enzyme.

Various experimental procedures were investigated to find suitable conditions to separate inactivated enzyme from excess cloxacillin and its hydrolyzed product, using low temperatures and methanol. The basic experiment was to inactivate enzyme by the standard incubation, at the completion of the inactivation to add methanol to 30%, and then to pass the sample through a centrifugal minigel column at low temperature. The standard procedure we had been using to maintain the pH constant in the incubation mixture was 0.5 M sodium phosphate buffer (recall that the inhibitor concentration was normally at least 200 mM). Unfortunately, sodium phosphate has limited solubility in aqueous methanol solvents, particularly at low temperatures. Consequently, it was necessary in these experiments to use a pH stat system to maintain the pH at

7 in the incubation system while at the same time minimizing the salt concentration. Several combinations of pH and temperature for the elution were investigated. The results demonstrated that at low pH (e.g., pH 4) very little activity was recovered, regardless of the temperature (0 to −20 °C).

Ultimately, the following conditions were found suitable to stabilize the inactivated enzyme sufficiently that negligible reactivation occurred over a 5–10-min period and that eventually 100% reactivation would ensue: incubation under standard conditions with the exception of using a pH stat rather than a buffer to maintain pH 7, addition of methanol to make the solution of 30% cosolvent by volume, passage through a pH 7 preequilibrated DEAE minigel by centrifugation at 10 °C for 2 min, and maintenance of pH 7, −10 °C.

Reactivation Monitored by Fluorescence. The tryptophan residues in *B. cereus* β-lactamase provide a sensitive probe for major conformational changes. Thus thermal or acid-induced unfolding are readily followed by changes in the intrinsic fluorescence emission of the enzyme (D. Joy and A. L. Fink, unpublished results). The cloxacillin-inactivated enzyme was separated from excess substrate and product by gel filtration at pH 7, as described above. Reactivation was then monitored at 0 °C either by repetitive scans of the fluorescence emission spectrum or by continuous monitoring at fixed wavelength. The reactivation was monitored simultaneously under identical conditions by a reactivation assay. No changes were observed in the emission spectra (λ_{max} or intensity) over the time period required for complete reactivation. The lack of change in the emission spectrum is good evidence that no significant change in the environment of the enzyme's tryptophans occurred in the inactivated enzyme.

These fluorescence experiments provided a very useful means of determining the concentration of β-lactamase present in the eluent from the centrifugal minigel chromatography. This was ascertained in experiments in which the intensity of the spectrum for the inactivated enzyme was compared to that of a control that had not been inactivated nor subjected to minigel chromatography. The ratio of intensities at the emission maximum was identical with the ratio of catalytic activities (after reactivation of the inactivated sample).

Susceptibility of Inhibited Enzyme to Trypsinolysis. Two different types of experiments were performed to compare the sensitivity of the inactivated and native enzyme toward trypsin proteolysis. In the first, the enzyme was incubated under standard conditions with cloxacillin or nafcillin until it was fully inactivated. Trypsin was then added to the incubation mixture, and after 15 min the sample was subjected to the reactivation assay. A control experiment in which the trypsin was omitted showed full reactivation (typically, 4 μM trypsin was added to 20 μM β-lactamase). The enzyme from the incubation mixtures containing cloxacillin and trypsin showed no reactivation, indicating that trypsin had caused sufficient proteolysis to render the enzyme nonactive. Similar results were obtained from experiments in which the enzyme had been inhibited with nafcillin. A further control in which the cloxacillin was omitted from the incubation mixture showed that the native enzyme, in the absence of cloxacillin, was resistant to trypsin, full activity being maintained under the comparable experimental conditions.

A more detailed picture of the effect of trypsin was obtained by using HPLC to assay for peptide products from trypsinolysis of the purified inactive enzyme. Incubation of trypsin for 15 min with β-lactamase alone, or in the presence of benzylpenicillin, showed no hydrolysis of the β-lactamase. Under these same conditions the inactivated enzyme, separated from excess inhibitor by gel filtration, showed substantial hydrolysis. Essentially no intact enzyme was present, and two new major peaks appeared with retention times of 10.6 and 13.2 min. (β-Lactamase and trypsin both eluted at 16.6 min in these experiments.) Smaller new peaks were observed at 4.2, 8.2, 9.4, 9.7, 9.9, 11.0, 12.2, 13.2, 14.2, and 17.4 min. Thus the cloxacillin-inactivated enzyme is orders of magnitude more sensitive to trypsinolysis than the native enzyme.

There are several possible explanations for the increased sensitivity to trypsin of the inactivated enzyme. One is that its conformation differs from that of the native enzyme, perhaps by exposure of a loop which is inaccessible to trypsin in the native enzyme. Such an exposed loop would be expected to provide access to trypsin. If the loop consisted of nonordered secondary structure, then it is possible that the difference in conformation between native and inactivated enzyme might not show up in the far-UV circular dichroism spectra. Another is that inactivation may be accompanied by separation of domains, or regions of secondary structure, leading to the exposure of trypsin-susceptible sites. An additional possibility is that the mobility of a region of the β-lactamase structure, perhaps a loop, is very different in the native and inactivated structures. Thus trypsin might have access to a scissile bond in the mobile state, and not in the less mobile form.

Circular Dichroism of Inactivated Enzyme. β-Lactamase (8.1 μM) was inactivated by cloxacillin (250 mM) under standard incubation conditions (pH 7.0) and freed from low molecular weight material by a DEAE-Sephadex minigel. The sample, in 30% methanol, pH* 7.0, was transferred to a cell in the spectropolarimeter at −10 °C. Scans from 240 to 195 nm were then taken at frequent intervals, while the temperature was maintained at −10 °C. Simultaneously, a reactivation assay was run on an aliquot of the same sample to monitor reactivation by the return of catalytic activity. Figure 5A shows a comparison of the CD spectrum of inactivated enzyme compared to that of the native enzyme under the same conditions. There is no significant difference in the spectra; therefore, we conclude that there are no significant changes in the secondary structure of the inactivated enzyme, compared to the native enzyme. The implications of this observation are discussed below. Figure 5B shows CD spectra prior to significant reactivation, after partial reactivation and after essentially complete reactivation. No significant change was observed in the CD spectrum over the time period corresponding to reactivation.

In preliminary circular dichroism experiments we had used a combination of low temperature and minigel chromatography at pH 4.5 to isolate and stabilize the inactivated enzyme, since incubation of the inactivated enzyme at low pH at 0 °C for 5 min had been shown to cause no irreversible inactivation. These experiments indicated that the inactivated enzyme had undergone a major conformational change (Figure 6). Since subsequent experiments under those conditions indicated that full reactivation did not occur at pH 4.5, we switched to pH 7, as noted above. In order to further investigate the initial observations at low pH, we isolated the inactivated enzyme in 30% methanol, pH* 7, −10 °C, as described above (and which shows a CD spectrum identical with that of the native enzyme), decreased the pH to 4.5, obtained the CD spectrum, readjusted the pH to 7, and collected the CD spectrum over a period of time. The CD spectrum of the inactivated enzyme when brought to pH 4.5, −10 °C, closely resembled that obtained by minigel chromatography at pH 4.5, 0 °C (Figure 6). The conformation is grossly different from that of the noninactivated enzyme under similar conditions, indicating that

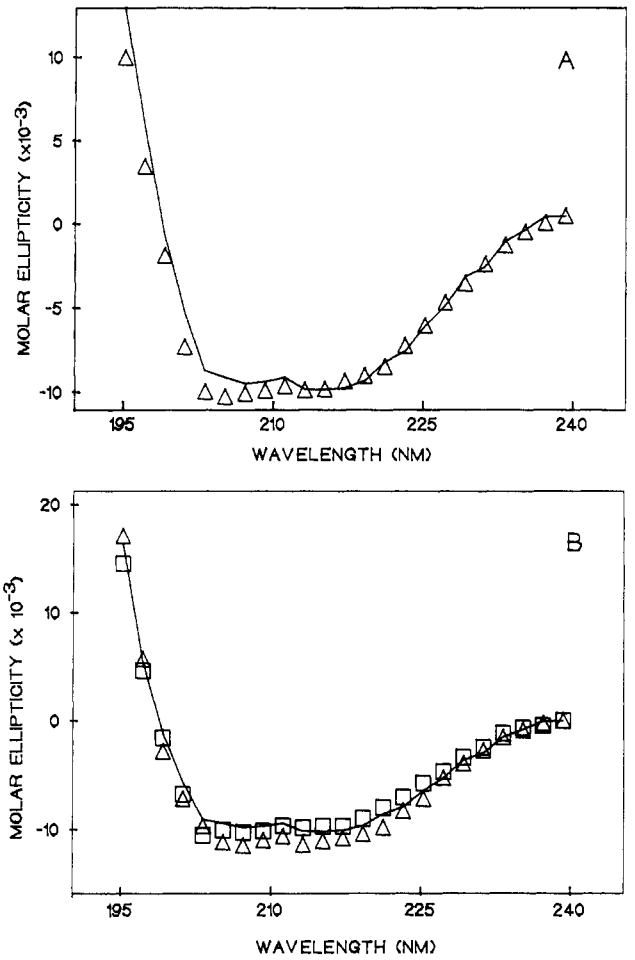
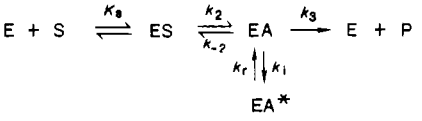


FIGURE 5: Circular dichroism spectra of native, inactivated, and reactivated β-lactamase. The enzyme (8.1 μM) was inactivated with cloxacillin (250 mM) and the inactivated enzyme separated from low molecular weight material at low temperature (−10 °C) by centrifugal ion-exchange chromatography, as described in the text. Panel A: Spectrum of the native enzyme in 30% methanol, pH* 7.0, −10 °C (△), compared to that of cloxacillin-inactivated enzyme under the same conditions (solid line). Data were collected at 2-nm intervals. Panel B: Spectrum of inactivated enzyme immediately after removal of substrate and product (solid line), after 5.5 h (72% reactivation) (□), and after essentially complete reactivation (95%) at 19 h (△). Molar ellipticities are in units of deg cm^2 dmol^{-1}.

Scheme I



pH values in the vicinity of 4–5 lead to major unfolding of the *inactivated* enzyme. Deconvolution of the CD spectrum for the inactivated enzyme at pH 4.5 indicates approximately two-thirds loss of the secondary structure. On return to pH 7 after 3 days of incubation at 5 °C the pH 4.5 treated sample showed negligible return of native structure or catalytic activity. The CD spectrum of the inactivated enzyme at pH 4.5 closely resembles that of the acid-denatured, unmodified enzyme at pH 2 (Figure 6). We believe that the acid-induced conformational transition is shifted to significantly higher pH for the inhibited form of the enzyme and leads to irreversible inactivation.

Kinetic Simulations and Models. We examine a number of models that might account for the observed *kinetic* properties of the enzyme–inhibitor system. The two most likely candidates are shown in Schemes I and II. In Schemes I and II EA represents the acyl-enzyme intermediate, EA* the inactivated enzyme, k_i the rate of inactivation, and k_r the rate of reactivation.

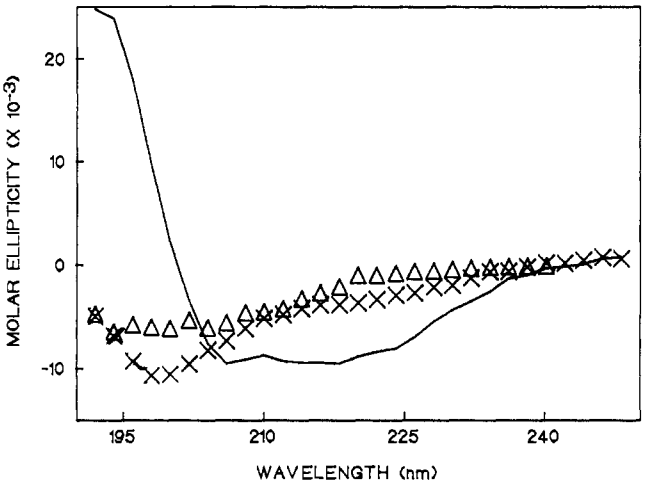
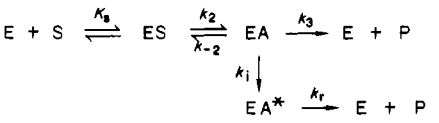


FIGURE 6: Circular dichroism spectra of native and inactivated β-lactamase at low pH. Both acid-denatured enzyme (pH 2.0) (×) and cloxacillin-inactivated enzyme at pH 4.5 (△) show drastic loss of secondary structure compared to the native enzyme at pH 4.5 (solid line). In this figure all spectra were obtained from aqueous buffer solutions at 0 °C. Due to uncertainties in the concentration of the cloxacillin-inactivated enzyme the data are reported as relative molar ellipticities.

Scheme II



Schemes I and II differ in that for the former the reactivation pathway is assumed to be the reverse of the inactivation and to involve the conversion of the inactive enzyme to the active acyl-enzyme. In Scheme II we assume that reactivation occurs via hydrolysis of the acyl-enzyme in the inactivated form of the enzyme, leading to formation of regenerated active enzyme, either directly or via an intervening isomerization involving the initial formation of enzyme in the same conformational state as in the inactivated enzyme complex (E*). Conversion of E* into E must be rapid since the rate of regeneration of catalytic activity on reactivation paralleled the rate at which cloxacillin penicilloate was produced, as determined by HPLC. It is unlikely that enzyme in the isomerized state, E*, could productively bind substrate and form E*S since the corresponding acyl-enzyme is inactive. Also, the rate of E* to E must be very fast so little free E* would be present. The possibility that ES can isomerize to E*S cannot be eliminated, but this seems unlikely to be a significant process.

The major difference in the two models is that the rate-limiting step in reactivation is a structural (conformational) change in Scheme I and hydrolysis (deacylation) of the inactivated acyl-enzyme in Scheme II. A reaction pathway, involving two forms of the enzyme, one much less active than the other, has previously been suggested by Virden et al. (1978), Kierner et al. (1980), and Frère (1981) to account for the observations with type A substrates.

Kinetically, Schemes I and II are equivalent and cannot be distinguished by kinetic properties alone. However, a number of our observations suggest that Scheme I is the better model. For example, the lack of significant pH dependence, lack of kinetic solvent isotope effect, and lack of effect of hydroxylamine on the rate of reactivation are all consistent with the rate-limiting step in reactivation which is structural, rather than chemical, in nature.

Schemes I and II were simulated by using appropriate values[2] for the rate constants. When necessary, the simulated data were then analyzed in the standard way used for the observed rates of inactivation and reactivation. The simulations showed that (1) substantial concentrations of inactivated enzyme only built up if deacylation was rate-limiting, (2) the concentration of inactivated enzyme was proportional to the ratio of rate of inactivation to reactivation, (3) the amount of inactivated enzyme produced was dependent on the concentration of inhibitor, up to some saturating value, and (4) the concentration of inactivated enzyme built up was also dependent on the ratio of the rate of deacylation to rate of inactivation. Furthermore, the observed K_d in plots of [EA*] against inhibitor concentration was considerably smaller than the value of K_s used in the simulation.

Source of Inactivation. How can we rationalize the observations that certain β-lactams act as both substrates and reversible inactivators of β-lactamase? First, the phenomenon is seen only with poor substrates, from which we infer that the deacylation step must not only be rate-limiting but also quite slow (less than about 100 s^{-1} at 25 °C, pH 7) compared to that of good substrates.[3] Second, there must be an alternative conformational state of the acyl-enzyme intermediate of comparable free energy to that with the enzyme in the native conformation. Thus substrates with slow deacylation steps provide sufficiently long-lived acyl-enzymes for the isomerization to the inactive conformational state to occur. Third, since reactivation occurs to regenerate the native enzyme, in the *absence* of bound substrate the more stable conformation of the free enzyme is the "normal" one. Fourth, we can infer a number of features about the structure of the inactivated enzyme. These are elaborated below.

There are two simple explanations why this type of reversible inactivation is not seen with good substrates such as benzylpenicillin. First, these substrates involve much faster rate constants for deacylation and hence much shorter lifetimes for the acyl-enzymes. In the case of cloxacillin the ratio of turnover to inactivation is about 3×10^3. Simulation of Scheme I for benzylpenicillin, using the same rates of inactivation and reactivation as found for cloxacillin, showed that less than 1.5% of the enzyme became inactivated with 1 mM substrate and 1 μM enzyme. Second, the data (Table I) for different inhibitors indicate that the rates of inactivation and reactivation are affected by the nature of the C6 side chain. Thus it is possible that the reactivation rate for good substrates (if EA* were formed) would be significantly faster than that for the poorer substrates. This would further minimize the concentration of inactivated enzyme.

Structure of the Inactivated Enzyme. Our results with fluorescence and far-UV circular dichroism on the isolated inactivated enzyme show that its conformational state cannot be too different from that of the normal native conformation, especially with respect to its secondary structure. From the fluorescence we can assume that the tertiary environment about the tryptophans is also unaffected. Recently, Phillips and co-workers (Samraoui et al., 1986) have published the secondary structure of this β-lactamase and Kelly et al. (1986) that of the closely related enzyme from *Bacillus licheniformis*. The overall fold of these enzymes resembles that of the R61 DD-peptidase, whose structure is available at higher resolution (Kelly et al., 1985). The major structural features are two "domains" or regions, one consisting of five helices, in which the active site serine is located, the other a five-stranded β-sheet with three associated helices. The active site of the peptidase has been located at the interface of these two domains, and it is reasonable, on the basis of amino acid sequence homology, to assume that the β-lactamase active site will be positioned similarly.

Both the change in susceptibility to trypsinolysis reported herein and previous reports concerning changes in H/T exchange rates (Kiener & Waley, 1977; Persaud et al., 1986) and effects of cross-linking by chemicals and antibodies (Carrey et al., 1984; Klemes & Citri, 1979; Citri et al., 1976) on inactivation by A-type substrates indicate that structural changes must occur on inactivation. The best way in which these observations can be reconciled is as follows. The circular dichroism data indicate that there cannot be any significant change in secondary structure in the inactivated form of the enzyme. Thus the changes in structure implied by the other techniques must involve movements of intact pieces of secondary structure, relative to each other. On the basis of the known low-resolution structure of the enzyme the two most likely explanations are either separation of the two domains or movement of one or more helix within the helical domain relative to the other helices. Both of these possibilities would be expected to affect the active site, perhaps, for example, moving a catalytic group involved in deacylation away from its required position.

The lack of effect on the fluorescence emission intensity in the inactivated enzyme could be explained by the putative location of the tryptophan residues. Since a high-resolution structure of β-lactamase is not presently available, the precise locations of the tryptophan residues are unknown. However, from our examination of the low-resolution structure of β-lactamase, and from comparison with the high-resolution structure of the R61 DD-peptidase, the Trp residues seem likely to be located far from the active site region in the vicinity of the B and C helices and the E β-strand in the "β-sheet" domain.

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Registry No. β-Lactamase I, 9001-74-5; cloxacillin, 61-72-3; methicillin, 61-32-5; nafcillin, 147-52-4.

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[2] Typical values were K_s = 1 mM, $k_2 = (1–10) \times 10^3$, $k_{-2} = 0$, $k_3 = 10–10^3$, $k_i = 1 \times 10^{-1}–1 \times 10^2$, and $k_r = 1–0.01k_i$; rates in units of s^{-1}.

[3] A-type substrates typically have values of $k_{cat} \leq 100$ s^{-1} (Citri et al., 1976).

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^{13}C NMR Studies of Porphobilinogen Synthase: Observation of Intermediates Bound to a 280 000-Dalton Protein†

Eileen K. Jaffe*,‡ and George D. Markham§

Department of Biochemistry, University of Pennsylvania School of Dental Medicine, Philadelphia, Pennsylvania 19104-6002, and Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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ABSTRACT: ^{13}C NMR has been used to observe the equilibrium complex of [4-^{13}C]-5-aminolevulinate ([4-^{13}C]ALA) bound to porphobilinogen (PBG) synthase (5-aminolevulinate dehydratase), a 280 000-dalton protein. [4-^{13}C]ALA (chemical shift = 205.9 ppm) forms [3,5-^{13}C]PBG (chemical shifts = 121.0 and 123.0 ppm). PBG prepared from a mixture of [4-^{13}C]ALA and [^{15}N]ALA was used to assign the 121.0 and 123.0 ppm resonances to C_5 and C_3, respectively. For the enzyme-bound equilibrium complex formed from holoenzyme and [4-^{13}C]ALA, two peaks of equal area with chemical shifts of 121.5 and 127.2 ppm are observed (line widths ~ 50 Hz), indicating that the predominant species is probably a distorted form of PBG. When excess free PBG is present, it is in slow exchange with bound PBG, indicating an exchange rate of $<10\ s^{-1}$, which is consistent with the turnover rate of the enzyme. For the complex formed from [4-^{13}C]ALA and methyl methanethiosulfonate (MMTS) modified PBG synthase, which does not catalyze PBG formation, the predominant species is a Schiff base adduct (chemical shift = 166.5 ppm, line width ~ 50 Hz). Free ALA is in slow exchange with the Schiff base. Activation of the MMTS-modified enzyme–Schiff base complex with ^{113}Cd and 2-mercaptoethanol results in the loss of the Schiff base signal and the appearance of bound PBG with the same chemical shifts as for the bound equilibrium complex with Zn(II) enzyme. Neither splitting nor broadening from ^{113}Cd–^{13}C coupling was observed.

Nuclear magnetic resonance (NMR)[1] has long been recognized as a potential probe of the active site chemistry of enzyme-catalyzed reactions. More than 10 years have passed since Mildred Cohn and co-workers first used ^{31}P NMR to investigate the equilibrium complex between substrates bound to an enzyme, arginine kinase (Rao et al., 1976). Although carbon chemistry far exceeds phosphorus chemistry in applicability to enzyme-catalyzed reactions, ^{13}C NMR as a probe of enzyme active sites suffers from the natural 1.1% isotopic abundance of ^{13}C. The low natural abundance dictates synthesis of isotopically enriched substrate molecules, while the high natural abundance contributes substantial resonance intensity (background) from the carbons of the protein. Nevertheless, Mackenzie et al. (1984) in a recent review predict a promising future for the use of ^{13}C NMR to study enzyme–substrate complexes below 50 000 daltons, using cryosolvents to prolong the lifetime of enzyme-bound intermediates.

Porphobilinogen (PBG) synthase (a.k.a. δ-aminolevulinate dehydratase, EC 4.2.1.24) catalyzes the asymmetric condensation of two molecules of 5-aminolevulinate (ALA) to form the pyrrole PBG. One ALA molecule forms the P (propionyl) side of PBG with its amino nitrogen being incorporated into the pyrrole ring, whereas the other ALA molecule forms the A (acetyl) side of PBG, retaining a free amino group (see Figure 1). This reaction is on the pathway for the biosynthesis of porphyrin, chlorophyll, vitamin B_{12}, and a wide spectrum of tetrapyrrole pigments. PBG synthase from bovine liver is an octameric protein of 280 000 daltons and thus might not be considered a promising candidate for investigation of enzyme–substrate complexes by ^{13}C NMR (Mackenzie et al., 1984). However, in a bisubstrate reaction with identical substrates, there are few potentially more powerful techniques for elucidating the chemistry of enzyme-bound reaction intermediates.

Current knowledge of the PBG synthase reaction mechanism is summarized below. The reaction proceeds via a Schiff base intermediate formed between one ALA and an active site lysine residue (Nandi & Shemin, 1968). The Schiff base forms to the C_4 of the ALA, which becomes the P side of PBG

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‡ University of Pennsylvania School of Dental Medicine.
§ Fox Chase Cancer Center.

[1] Abbreviations: ALA, 5-aminolevulinate; NMR, nuclear magnetic resonance; MMTS, methyl methanethiosulfonate; PBG, porphobilinogen; EDTA, ethylenediaminetetraacetic acid.

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