β-Lactamase Proceeds via an Acyl-Enzyme Intermediate. Interaction of the *Escherichia coli* RTEM Enzyme with Cefoxitin†

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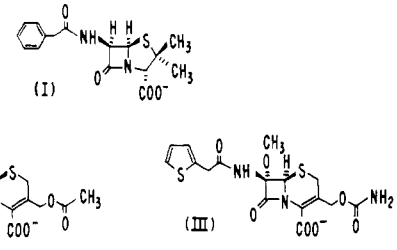
ABSTRACT: The use of cefoxitin, a poor substrate of the RTEM β-lactamase, has allowed the kinetic and spectroscopic characterization of a covalent acyl-enzyme intermediate in the enzyme-catalyzed reaction. The rate of reappearance of catalytic activity in an enzyme sample diluted from an incubation with cefoxitin is nearly identical with the observed k_{cat}. Burst kinetics are observed with this substrate, consistent with the rate-limiting deacylation of the cefoxitinoyl-enzyme. That the reaction intermediate involves a covalent link between enzyme and substrate was shown by gel filtration after rapid denaturation of an enzyme–[^{14}C]cefoxitin reaction at the steady state. Fourier transform infrared measurements indicate that the intermediate is an acyl-enzyme involving a hydroxyl group of the β-lactamase. The evident relationship between the acylation–deacylation sequence of the β-lactamases and the acylation reaction suffered by the D-Ala-D-Ala-carboxypeptidases is discussed.

The acquisition of the capacity to produce a β-lactamase is probably the most common reason for resistance to β-lactam antibiotics in bacteria (Richmond et al., 1971; Abraham, 1977; Fisher & Knowles, 1978). β-Lactamases catalyze the hydrolytic opening of the β-lactam ring, and so convert the antibiotic into an innocuous product that can no longer interfere with the synthetic machinery of the bacterial cell wall. The relentless growth in the number of resistant bacterial strains and the development of whole new classes of nonclassical β-lactam antibiotics to combat them (Cama & Christensen, 1978) have focused attention on β-lactamase as an important target in the effort to retain and extend the therapeutic utility of the β-lactams as antibiotics.

The β-lactamases have, rather surprisingly, attracted little mechanistic scrutiny, and this lack of information has undoubtedly hindered the rational development of reagents that will inactivate these enzymes. Even the most basic question that one may ask concerning a hydrolytic enzyme of this type has not been answered. Does the enzyme act as a general acid–base catalyst to facilitate the direct attack of a water molecule on the β-lactam carbonyl group, or does it instead provide a nucleophile to attack the β-lactam, leading to formation of an acyl-enzyme intermediate? It is to this fundamental question that the present work is addressed.

The intermediacy of acyl-enzymes in the peptidolytic reactions catalyzed by such enzymes as α-chymotrypsin, elastase, and papain has been established unequivocally. Acyl-enzymes that occur *before* the rate-limiting transition state have been observed directly, isolated, and characterized, and their chemical and kinetic competence has been demonstrated [see, e.g., Kraut (1977) and Lowe (1970)]. While the existence of acyl-enzymes that occur *after* the rate-limiting transition state is harder to prove, trapping experiments (using, for instance, the classical criterion of "product diversion with no change in rate") have been successful in many cases [see, e.g., Fastrez & Fersht (1973)]. For β-lactamases, none of these approaches has yielded evidence in support of an acyl-enzyme, and, largely by default, support for the general base postulate has persisted [see, e.g., Thatcher (1975)]. Yet the substrates normally studied have been excellent, having values of k_{cat}/K_m around 10^8 M^{-1} s^{-1}, which is close to the diffusion limit. It may be that these good substrates (such as benzylpenicillin or phenoxymethylpenicillin) have kinetic characteristics that preclude the observation of reaction intermediates, the lifetime of which would be short and the steady-state concentration of which would be small. Accordingly, to increase the chance of detecting intermediates along the reaction pathway, we have chosen to investigate the behavior of a *poor* substrate for the β-lactamase.

The β-lactamase used in this work, the plasmid-encoded RTEM-2[1] enzyme, catalyzes the hydrolysis of benzylpenicillin (I) with a k_{cat}/K_m of 10^8 M^{-1} s^{-1} (k_{cat} = 2000 s^{-1}; K_m = 20 μM). Cephems are poorer substrates, cephalothin (II) having a k_{cat}/K_m of 6×10^5 M^{-1} s^{-1} (k_{cat} = 120 s^{-1}; K_m = 200 μM). The introduction of a 7-α-methoxy substituent, as in cefoxitin (III) (Karady et al., 1972), causes a sharp drop in the rate of hydrolysis, and k_{cat}/K_m falls to 6 M^{-1} s^{-1} (k_{cat} = 0.0040 s^{-1}; K_m = 650 μM). Cefoxitin is a representative of the cephamycin group of antibiotics, which not only retain the antibacterial spectrum of the cephems, but extend it to many cephalosporin-resistant Gram-negative bacteria (Cama & Christensen, 1978). The 7-α substituent selectively reduces the lability of the β-lactam to β-lactamase action (Onishi et al., 1974) without having a significant effect on the affinity



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[1] Abbreviations used: RTEM specifies the source of the plasmid [see Datta & Kontomichalou (1965)] and TEM-2 specifies hl. enzyme [see Sutcliffe, (1978)]. The wild-type enzyme is designated wt, and the mutant enzyme reported in Hall & Knowles (1976) is designated hl.

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for the membrane penicillin binding proteins (Spratt, 1977; Matsuhashi & Tamaki, 1978).

We report here the interaction of cefoxitin with the RTEM β-lactamase, and show for the first time that (for this substrate at least) the enzyme-catalyzed reaction proceeds via the formation of an acyl-enzyme intermediate.

Experimental Procedures

Materials

Sodium cefoxitin and sodium [^{14}C]*cefoxitin* were generous gifts from Merck Sharp & Dohme. The concentration of stock solutions was determined from the absorbance at 261 nm (ϵ = 9250 M^{-1} cm^{-1}). The radiolabeled cefoxitin (6690 dpm $nmol^{-1}$) contained ^{14}C specifically at the thienyl acetate carbonyl carbon.

β-Lactamase. Two β-lactamases were used in this study: the wild-type (wt) TEM-2 β-lactamase (Ambler & Scott, 1978; Sutcliffe, 1978) identical with that used in our studies with clavulanate (Fisher et al., 1978), and a mutant β-lactamase (h1) derived from wild-type enzyme (Hall & Knowles, 1976). The nature of the point mutation in h1 is not yet known. The wt and h1 β-lactamases were purified to homogeneity and had specific activities of 68.3 and 23.3 μkat A_{281}^{-1} (benzylpenicillin as substrate at pH 7.0, 30 °C), respectively. All the kinetic and chemical experiments, save where indicated, were done in 0.10 M sodium or potassium phosphate buffer (pH 7.0) at 30 °C. The ultraviolet absorption spectra of these two enzymes are identical, with an extinction coefficient at 281 nm of 29 400 M^{-1} cm^{-1}. This extinction coefficient differs considerably from the previously accepted value (21 400 M^{-1} cm^{-1}; Melling & Scott, 1972) and reflects the presence of four tryptophan and four tyrosine residues (rather than three apiece) in the amino acid sequence (Ambler & Scott, 1978; Sutcliffe, 1978).

The β-lactamases were purified by a new procedure that takes advantage of the enzymes' location in the *Escherichia coli* periplasmic space (Charm & Matteo, 1972). *E. coli* W3310 carrying the plasmid RP1 (320 g, wet weight) was washed with 50 mM Tris-HCl buffer, pH 7.0, and then suspended for 30 min at room temperature in 25 mM Tris-HCl buffer, pH 7.0 (1 L), containing sucrose (450 g) and Na_4-EDTA (0.5 g). The bacteria were isolated by centrifugation and the supernatant was discarded. The cells were quickly dispersed in cold (5 °C) distilled water (1.5 L) for 30 min with vigorous stirring. After centrifugation, the supernatant, containing the β-lactamase (5.7 mkat, specific activity 1.2 μkat A_{281}^{-1}), was recovered. The supernatant was brought to 10 mM in Tris-HCl, pH 6.75, by the addition of concentrated (1 M) buffer, and the solution was then applied to a column (400 mL) of DEAE-cellulose (DE-52) equilibrated with 10 mM Tris-HCl buffer, pH 6.75. The enzyme barely adhered to this column, and the first 2 L of effluent was collected, containing 5.0 mkat of β-lactamase having a specific activity of 16.7 μkat A_{281}^{-1}. The buffer concentration was brought to 15 mM by the addition of 100 mM triethanolamine-HCl buffer, pH 7.25, and the pH of the combined solutions was adjusted to pH 7.25 by the addition of NaOH (2 M). The solution was then applied to a second DE-52 column (2.5 × 28 cm) equilibrated with 15 mM triethanolamine-HCl, pH 7.25. This column was developed with a linear gradient (750 mL + 750 mL) of triethanolamine-HCl buffer, pH 7.25 (15 to 100 mM). The β-lactamase eluted halfway through the gradient, and those fractions having a specific activity greater than 33 μkat A_{281}^{-1} were pooled. The pool contained 4.3 mkat at a specific activity of 51.7 μkat A_{281}^{-1}. The enzyme solution was concentrated to 4 mL by ultrafiltration and applied in two equal portions to a column (2.5 × 52 cm) of Sephadex G-100. The two sets of peak fractions having specific activities greater than 66 μkat A_{281}^{-1} were pooled. The side fractions were rechromatographed on Sephadex G-100.

The final yield of β-lactamase was 2.03 μmol (4.1 mkat, 68.3 μkat A_{281}^{-1}). On the rare occasions when the specific activity at this point was less than 66 μkat A_{281}^{-1}, the purification was completed by subjecting the β-lactamase to further chromatography on a column (1.5 × 10 cm) of DE-52 equilibrated with 10 mM potassium phosphate buffer, pH 7.0, and eluting with this same buffer. Purified enzyme was stored in solution at 4 °C with 10 mM NaN_3 as a bacteriocide for periods of several weeks, and for longer periods as a freeze-dried powder.

Methods

All kinetic experiments were done at 30 °C in 0.10 M sodium phosphate buffer, pH 7.0, unless otherwise stated. Ultraviolet measurements were made on a Perkin-Elmer 575 spectrophotometer. Scintillation counting was done on a Beckman LS-233 instrument, using a dioxane-based scintillation fluid [950 mL of dioxane, 100 g of naphthalene, and 8.0 g of Omnifluor (New England Nuclear)]. The fluid was calibrated with a [^{14}C]hexadecane standard containing various amounts of water, and the disintegrations per minute were calculated from the observed counts per minute using these calibration factors and the external standard ratio.

Infrared measurements were made at 2-cm^{-1} resolution using a Nicolet 7199 Fourier transform infrared spectrometer purged with N_2. The sample cell had CaF_2 windows and a Teflon spacer (0.107 mm) and was mounted on a water-jacketed cell holder maintained at 25 °C. The solvent was D_2O. Measurements of pH were made at room temperature with a Radiometer PHM64 pH meter and a Microelectrodes, Inc. MI-410 combination electrode. Reported pD values are 0.4 unit higher than those actually read on the pH meter (Glasoe & Long, 1960).

Quantitation of the Spectral Changes Accompanying Cefoxitin Hydrolysis. The hydrolytic cleavage of a cephalosporin β-lactam occurs with a substantial decrease in the absorption band centered near 260 nm (Ross & O'Callaghan, 1975). The extinction coefficient change in the case of cefoxitin was calculated from the extinction coefficient of the intact cefoxitin and that of cefoxitin after complete enzymatic hydrolysis at pH 7.0. At 261 nm, $\Delta\epsilon$ was 7775 M^{-1} cm^{-1} and at 290 nm it was 1600 M^{-1} cm^{-1}. For comparison, cephalothin has $\Delta\epsilon_{265}$ = 7700 M^{-1} cm^{-1}.

Values of k_{cat} and K_m were obtained from Eadie–Hofstee plots of the initial steady-state velocities of hydrolysis at various cefoxitin concentrations. The reaction mixture (0.30 mL in a 1.0-mm path length cuvette) contained enzyme (5.1 nmol, final concentration 17.0 μM) and cefoxitin (initial concentrations of 0.15 to 3.88 mM for the wt enzyme, 0.058 to 1.91 mM for the h1 enzyme). Spectral changes at 30 °C were followed at either 261 or 290 nm. The validity of the kinetic parameters based upon initial velocity measurements was confirmed by fitting the integrated Michaelis–Menten equation (Gutfreund, 1972) to the complete reaction progress curve at 261 nm. The agreement between the parameters determined by the two methods showed that the intramolecular elimination from the cephalosporoic acid known to occur immediately after β-lactam opening (Ross & O'Callaghan, 1975; Boyd & Lunn, 1979) does not affect the determination of the steady-state kinetic parameters. Since the initial velocity method yields more reliable values, the k_{cat} and K_m values used here are those derived by this method.

Determination of the Deacylation Rate Constant. The rate constant for deacylation of the cefoxitinoyl-enzyme was obtained by diluting a sample from an incubation mixture of enzyme saturated with cefoxitin into an assay cuvette containing a large excess of benzylpenicillin. The instantaneous velocity for benzylpenicillin hydrolysis following this dilution is directly proportional to the amount of free enzyme present. Enzyme (10 μL of the wt enzyme containing 0.146 nmol) was added to buffered cefoxitin (200 μL of 16.2 mM). After 20 min a portion (10 μL) was withdrawn and added to the assay cuvette containing benzylpenicillin (3.0 mL of 2.0 mM). The hydrolysis of benzylpenicillin was followed at 240 nm (Samuni, 1975). Cefoxitin was present in the assay cuvette at a final concentration of 51 μM, 14-fold lower than its K_m, and thus did not competitively inhibit the hydrolysis of benzylpenicillin, which was present at a concentration 100 times its K_m. The rate constant for the first-order increase in the rate of benzylpenicillin hydrolysis was determined from the progress curve by the method of Glick et al. (1978).

Observation of the Pre-Steady-State Burst. The measurement of the "burst" rate was made as follows: To a 10.0-mm path length cuvette was added a solution of the wt enzyme (476 nmol, in 700 μL). The reaction was initiated by the addition of cefoxitin (3.0 μmol, in 100 μL). The molar ratio of cefoxitin to enzyme is therefore initially 6.3. A quartz block, 9.50 × 8.00 mm with optical faces on all four sides, was inserted so as to shorten the path length to 0.50 mm. The progress curve at 261 nm was then obtained. The rate constant and burst size were determined by applying the double Guggenheim method (Gutfreund, 1972) and then correcting for the fact that cefoxitin was not at a saturating concentration. For the h1 enzyme, the reaction mixture contained enzyme (72 nmol) and cefoxitin (670 nmol) in a final volume of 600 μL of 0.10 M potassium phosphate buffer, pH 7.0, in a 2.0-mm path length cuvette.

Failure of Hydroxylamine to Accelerate Turnover by Acyl-Enzyme Trapping. The h1 β-lactamase was used for this experiment, since it is possible to saturate the enzyme at lower cefoxitin concentrations and thus to minimize the rate of hydroxylaminolysis of free cefoxitin. The rate of change of A_{261} was determined in a 1.0-mm path length cuvette, containing in 0.30 mL of buffer the h1 enzyme (43 μM), NH_2OH (48 mM), and cefoxitin (1.9 mM). Hydroxylamine does not cause any loss in enzymatic activity under these conditions.

Isolation of the Acyl-Enzyme. [^{14}C]Cefoxitin (3.33 μmol) was dissolved in a buffered solution (350 μL) of the wt β-lactamase (20.7 nmol), and the mixture was incubated at 30 °C. After 20 min, the mixture was cooled quickly to 0 °C and applied to a column (1.5 × 15 cm) of Sephadex G-25 supefine at 0 °C. The column was developed at a flow rate of 17.5 mL h^{-1} with ice-cold 25 mM potassium phosphate buffer, pH 7.0. Fractions of 0.875 mL were collected. Three fractions containing enzyme were collected and contained 0.82, 0.75, and 0.86 mol of cefoxitin per mol of enzyme. One of these fractions was warmed to 30 °C for 15 min and then resubjected to gel filtration. The enzyme fractions from this second column showed that more than 90% of the radioactivity that had migrated with the enzyme on the first column no longer coeluted.

Covalent Nature of the Acyl-Enzyme. [^{14}C]Cefoxitin (1.44 μmol) was dissolved in a buffered solution (100 μL) of the wt enzyme (10.7 nmol) and incubated at 30 °C. After 20 min the reaction was quenched by the addition of a solution (150 μL) of sodium dodecyl sulfate (40 mM) and vortexed vigorously for 5 min. The solution was applied to a column (1.5 × 18 cm) of Sephadex G-25, equilibrated at room temperature with 50 mM sodium phosphate buffer, pH 7.0, containing sodium dodecyl sulfate (20 mM). The column was developed at a flow rate of 26.4 mL h^{-1}, and fractions of 1.1 mL were collected. The radioactivity migrating with the denatured enzyme was in a stoichiometry of 1.4 mol per mol of enzyme. This high value resulted from a failure to achieve a base-line separation, together with some adventitiously adsorbed cefoxitin. Dialysis against aqueous sodium dodecyl sulfate at neutral pH resulted in material containing 1.0 mol of cefoxitin per mol of enzyme. In a control experiment, the above sequence was repeated but with enzyme denaturation preceding the addition of the radiolabeled cefoxitin. Only 0.3 equiv of cefoxitin coeluted with the denaturated enzyme, and this fell below 0.1 equiv following dialysis.

Table I: Kinetic Parameters for the Hydrolysis of Cefoxitin by the Wild-Type and Mutant h1 β-Lactamases

	wild-type enzyme		h1 enzyme	
	obsd	calcd	obsd	calcd
k_{cat} (s^{-1})	4.0 × 10^{-3}		4.1 × 10^{-3}	
K_m (mM)	0.65		0.10	
k_3 (s^{-1})	4.8 × 10^{-3}		4.7 × 10^{-3}	
k_2 (s^{-1})		2.4 × 10^{-2}		3.2 × 10^{-2}
K_s (mM)		3.9		0.78
k_{obsd} (s^{-1})	1.6 × 10^{-2}	1.7 × 10^{-2}	2.5 × 10^{-2}	2.4 × 10^{-2}
$\pi/[e]_0$	0.34	0.50	0.66	0.64

Infrared Spectra of Cefoxitin and of the Acyl-Enzyme. "Double-beam" infrared spectra of cefoxitin during the course of its enzyme-catalyzed hydrolysis in D_2O were obtained by the ratio, at various extents of reaction, of single-beam spectra of sodium cefoxitin (initially 20.2 mM) in the presence of the wt β-lactamase (8.2 mM) in 161 mM sodium phosphate buffer (pD 7.7), against a single-beam spectrum of the same sample after completion of the hydrolysis. For each single-beam spectrum, 117 interferograms were averaged, requiring an acquisition time of 2.0 min. The concentrated solution of β-lactamase in D_2O was prepared by exhaustively dialyzing the enzyme against 2 mM sodium phosphate buffer, pH 7.0, freeze-drying the sample, and redissolving it in a minimum of D_2O.

Results

The experimental results presented here describe the kinetic, chemical, and spectroscopic evidence for an acyl-enzyme intermediate during the hydrolysis of cefoxitin by the wild-type TEM-2 β-lactamase. A second enzyme, the h1 TEM-2 β-lactamase (Hall & Knowles, 1976), has also been examined. The h1 enzyme is a mutant of the wild type having enhanced cephalosporinase activity, and the kinetic parameters of this mutant enzyme are presented with those of the wild-type enzyme in Table I. The experiments described below (save for a single experiment) refer, however, to the wild-type enzyme.

Determination of k_{cat} and K_m. Cleavage of the β-lactam ring of a cephalosporin results in the almost complete disappearance of the intense absorption centered near 260 nm (Ross & O'Callaghan, 1975). The enzyme-catalyzed hydrolysis of cefoxitin was therefore followed at 261 nm. The reaction is extremely slow, but follows Michaelis–Menten kinetics, with a k_{cat} of 4.0 × 10^{-3} s^{-1} and a K_m of 0.65 mM.

Biphasic Kinetics. When β-lactamase is incubated with a saturating concentration of cefoxitin and allowed to reach the steady state, dilution of a small portion of this mixture into an assay cuvette containing an excellent substrate for the β-lactamase gives an initial rate for the good substrate's hy-

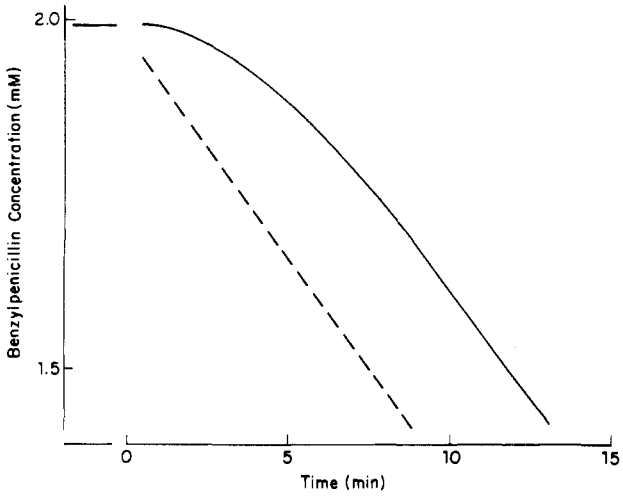

FIGURE 1: Hydrolysis of benzylpenicillin catalyzed by wild-type β-lactamase: (solid line) enzyme portion taken from an incubation containing a saturating concentration of cefoxitin; (dashed line) enzyme portion taken from a control stock solution containing no cefoxitin.

Scheme I: Three-Step Scheme for Hydrolysis of Cefoxitin (c) by the β-Lactamase (e) via a Michaelis Complex (e·c) and Reaction Intermediate (the Proposed Acyl-Enzyme) (e–c)

$$e + c \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} e{\cdot}c \xrightarrow{k_2} e\text{–}c \xrightarrow{k_3} e + p$$

drolysis that is close to zero. The rate then accelerates in a first-order manner over several minutes, finally reaching a limiting rate that is identical with what would have been observed immediately had cefoxitin not been present in the first incubation (Figure 1). The instantaneous rate of substrate hydrolysis reflects the amount of free enzyme that is present. Both the rate of reappearance of enzyme activity and the final reaction rate are independent of the duration of the incubation with cefoxitin after the steady state has been reached. Two conclusions can be drawn from these results. First, since the terminal velocity is that for fully active enzyme, cefoxitin is not an inactivator of the enzyme. Second, the similarity between the rate constant for activity regain (4.8×10^{-3} s^{-1}) and the k_{cat} for cefoxitin (4.0×10^{-3} s^{-1}) suggests that during the enzyme-catalyzed hydrolysis of cefoxitin, a reaction intermediate accumulates whose breakdown is rate determining. The catalytic pathway is represented in Scheme I, where $k_2 > k_3$.

Since the rate constant for activity regain is k_3, we may calculate, on the basis of Scheme I, the value of k_2 from the knowledge that $k_{cat} = k_2k_3/(k_2 + k_3)$. Given also the relatively small magnitudes of k_2 and k_3 and the large K_m, it is safe to assume that k_2 and k_3 are both much smaller than k_{-1}, which allows the estimation of K_s ($= k_{-1}/k_1$) from $K_m = (k_{-1} + k_2)k_3/(k_2 + k_3)k_1$. The derived values of k_2 and K_s are listed in Table I.

The pathway of Scheme I, with $k_2 > k_3$, predicts that, on the addition of enzyme to cefoxitin, there should be a "burst" of cefoxitin consumption as e–c is rapidly formed. To test for such a burst, the absorbance at 261 nm was followed from the moment of mixing a relatively high concentration of wild-type enzyme (0.585 mM) with cefoxitin (4.0 mM). The trace obtained (Figure 2) showed the anticipated burst. Under the conditions used, the observed rate constant for the approach to the steady-state rate of enzymatic hydrolysis was 1.6×10^{-2} s^{-1}, and the extrapolated magnitude of the burst corresponded to 0.34 molar equiv of enzyme. For systems that obey Scheme I, the expressions for the burst rate constant (k_{obsd}) and the

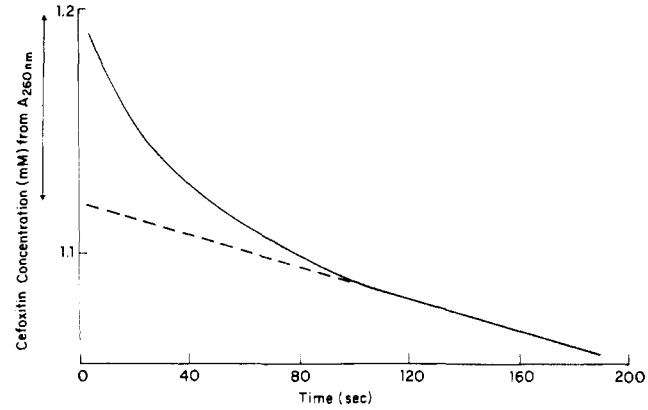

FIGURE 2: Hydrolysis of cefoxitin catalyzed by the mutant hl β-lactamase. The arrow on the ordinate shows the size of the burst.

burst size ($\pi/[e]_0$) are given by the following equations (Kezdy & Bender, 1962; Bender, 1972):

$$k_{obsd} = \frac{(k_2 + k_3)[s]_0 + k_3K_s}{K_s + [s]_0} \quad (1)$$

$$\pi/[e]_0 = \frac{[k_2/(k_2 + k_3)]^2}{[1 + K_m/[s]_0]^2} \quad (2)$$

The values of k_{obsd} and $\pi/[e]_0$ observed in the burst experiment, and calculated from the kinetic parameters derived from the experiments described earlier, are compared in Table I. The gratifying agreement between the observed and calculated quantities confirms that Scheme I adequately describes the kinetic sequence of cefoxitin hydrolysis by the β-lactamase. Several assumptions implicit in this scheme are also justified. The decomposition of e–c may be taken as irreversible (i.e., $k_{-3} = 0$), since hydrolyzed β-lactams (penicilloic acids and cephalosporoic acids) are poorly bound by the β-lactamase (Kiener & Waley, 1978). The assumption that the formation of e–c is also effectively irreversible (i.e., that $k_3 > k_{-2}$) is supported by the substantial free-energy change that accompanies β-lactam ring opening and by the results of the infrared examination of the hydrolysis of cefoxitin (see below).

It should be pointed out, however, that the burst experiment cannot be done under the ideal condition of a substrate concentration very much greater than both K_m and the enzyme concentration. The enzyme concentration must be high enough for the burst of absorbance to be observable, yet since a *decrease* in absorbance is being followed, there is a practical limit to the substrate concentration. The conditions used for the experiment of Figure 2 represent a reasonable compromise of these constraints and allow the nonstoichiometric burst to be analyzed theoretically.

Finally, since the reaction intermediate (e–c in Scheme I) is believed to be an acyl-enzyme (see below), an attempt was made to accelerate its breakdown by the addition of hydroxylamine as a more nucleophilic surrogate for water. Under the conditions described under Methods, the rate of the enzyme-catalyzed reaction is 0.403 A_{261} h^{-1}. When hydroxylamine is added, the rate of substrate loss rises to 0.912 A_{261} h^{-1}, but this rise of 0.509 A_{261} h^{-1}, is accounted for by the *non*-enzyme-catalyzed reaction of hydroxylamine with the β-lactam (measured independently to be 0.473 A_{261} h^{-1}). It is evident from these data that hydroxylamine does not significantly accelerate the rate of the enzyme-catalyzed reaction.

Isolation of the Reaction Intermediate. The above experiments have established that a reaction intermediate (e–c in Scheme I) is formed during the hydrolysis of the cefoxitin by β-lactamase and that its rate of formation is greater than its

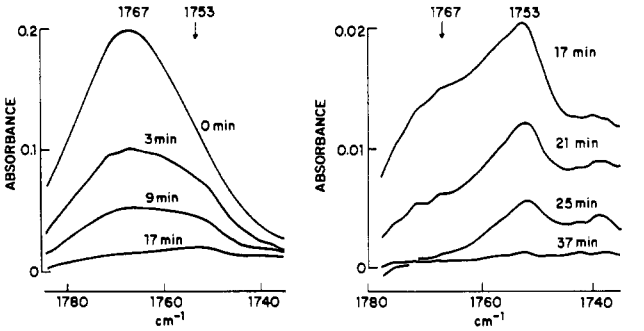

FIGURE 3: Fourier transform infrared spectra of the hydrolysis of cefoxitin by the wild-type β-lactamase. The change in ordinate scale should be noted (the top curve of the right-hand group of spectra is equivalent to the bottom curve of the left-hand group of spectra). Initial cefoxitin concentration, 20.2 mM; enzyme concentration, 8.2 mM.

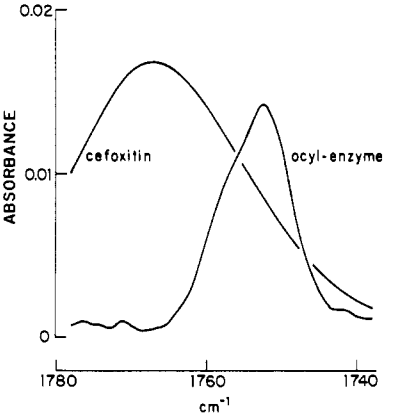

FIGURE 4: Fourier transform infrared spectra of cefoxitin and of the acyl-enzyme formed from cefoxitin and the wild-type β-lactamase. The latter spectrum was obtained by subtraction of a spectrum of cefoxitin from a spectrum of the β-lactamase–cefoxitin reaction mixture at t = 5 min, to give a flat base line.

rate of breakdown. It was therefore feasible to isolate this intermediate and to investigate whether a *covalent* link was responsible for the attachment of the substrate to the enzyme. First, the wild-type enzyme was combined with a saturating concentration of [^{14}C]cefoxitin and incubated at 30 °C until well after the steady state had been achieved (~20 min). The solution was rapidly cooled to 0 °C and subjected to gel filtration at this temperature. The protein fractions contained approximately 0.81 mol of cefoxitin (determined from ^{14}C) per mol of enzyme (determined from the catalytic activity after incubation at 30 °C; see Figure 1). That this species was indeed the reaction intermediate was shown from its behavior when diluted into an assay solution of benzylpenicillin in an experiment analogous to that shown in Figure 1. After a sample of the complex was warmed to 30 °C for 20 min, gel filtration showed that more than 90% of the ^{14}C had been lost from the enzyme. To demonstrate that cefoxitin is covalently linked to the enzyme in the complex, a known amount of enzyme in solution with a saturating concentration of cefoxitin was denatured with sodium dodecyl sulfate and then subjected to gel filtration and dialysis in the presence of denaturant. Under these conditions, 1.0 mol of cefoxitin remains attached per mol of enzyme. These experiments demonstrate that in the isolated reaction intermediate cefoxitin is covalently bound, and that formation of the free enzyme from this complex during the overall reaction is enzyme catalyzed.

Nature of the Reaction Intermediate. Is the intermediate an acyl-enzyme? From the fact that the formation of this intermediate is accompanied by a decrease in the absorbance at 261 nm, we can conclude that the β-lactam ring has evidently been lost in the covalent attachment of cefoxitin to the enzyme. While this is suggestive of enzyme acylation, more direct evidence was obtained from the infrared spectrum of the complex itself. Using Fourier transform infrared spectrophotometry, it is possible to obtain the partial spectrum of a species such as the intermediate in the β-lactamase reaction, where one is looking for the absorption of a single group (in this case, an ester) on a protein molecule of molecular weight 29 000. When the hydrolysis of cefoxitin by a nearly stoichiometric amount of the wild-type β-lactamase was followed by infrared spectroscopy, two absorption bands were observed between 1720 and 1800 cm^{-1}, which decayed at different rates (see Figure 3 and 4). One of these, at 1767 cm^{-1} (ϵ 0.09 mM^{-1} mm^{-1}), corresponded to the β-lactam carbonyl absorption of cefoxitin itself. The other, at 1753 cm^{-1}, was not present in the spectra of cefoxitin, of the unliganded enzyme, or of the lactam hydrolysis product. The band at 1753 cm^{-1} was in the region expected for an α-methoxy ester, with an appropriate extinction coefficient for such a functionality (ϵ ~0.02 mM^{-1} mm^{-1}). After the disappearance of the cefoxitin band, the absorption at 1753 cm^{-1} decayed with a first-order rate constant of 3.9 ± 0.4 × 10^{-3} s^{-1}. [In addition, a negative peak centered at 1707 cm^{-1} disappeared with time. By the method used to obtain "double beam" spectra, this corresponds to an accumulating hydrolysis product.] Similar results were obtained with the mutant h1 enzyme.

That the band at 1753 cm^{-1} did not represent a slowly hydrolyzing impurity in the cefoxitin sample is evidenced by the fact that its maximum intensity increased with increasing enzyme concentration. Moreover, the narrow half-width (11 cm^{-1}, compared with 26 cm^{-1} for the β-lactam carbonyl of free cefoxitin) is characteristic of an enzyme-bound species. The possibility that the band at 1753 cm^{-1} was an absorption of the Michaelis complex of cefoxitin bound to the enzyme is ruled out by the fact that this absorption persisted after the disappearance of the cefoxitin band. Since the dissociation constant of the e·c complex is large, such behavior is only consistent with a species absorbing at 1753 cm^{-1} which is formed irreversibly from cefoxitin. Finally, the first-order rate constant for the decay of this band (3.9 ± 0.4 × 10^{-3} s^{-1} in D_2O at 25 °C) is close to the deacylation rate constant (k_3) determined independently under the conditions of solvent, buffer, pD, and temperature employed in the infrared experiment (2.8 ± 0.7 × 10^{-3} s^{-1}). The most reasonable explanation for the location and kinetic behavior of this absorption is therefore that it derives from an acyl-enzyme intermediate in the β-lactamase-catalyzed hydrolysis of cefoxitin.

Discussion

The experiments described here demonstrate that the hydrolysis of cefoxitin catalyzed by the RTEM-2 β-lactamase proceeds via an intermediate that involves a covalent bond between enzyme and substrate with spectroscopic features characteristic of an ester linkage (i.e., an acyl-enzyme). We have argued in the introductory statement that the chances of observing an acyl-enzyme are highest if the intermediate forms more quickly than it collapses, and for that reason a very poor substrate, cefoxitin, was chosen for scrutiny. The question of whether acyl-enzymes are intermediates in the β-lactamase-catalyzed hydrolysis of *all* substrates cannot yet be answered, since it appears that for the more rapidly hydrolyzed β-lactam substrates no intermediate accumulates. But the suggesion that the RTEM β-lactamase universally follows an acyl-enzyme pathway does not rest simply on the cefoxitin

results and a belief in mechanistic economy. For example, the 6α-substituted carbapenem thienamycin (Cama & Christensen, 1978) also shows biphasic hydrolysis kinetics that are most readily interpreted in terms of the formation of an acyl-enzyme (J. Fisher, unpublished experiments). Furthermore, the interaction of the enzyme with all the known β-lactamase-inactivating reagents [e.g, clavulanic acid, 6β-bromopenicillanic acid, penicillanic acid sulfone, 8-sulfatoolivanic acid, 6-chloropenicillanic acid sulfone, etc. (for a summary, see Fisher et al., 1980)] is most readily interpreted in terms of the initial formation of an acyl-enzyme that may either suffer hydrolysis (like a simple substrate) or some other fragmentation (which may result in enzyme inactivation). For example, 6β-bromopenicillanic acid inactivates the β-lactamase I from *Bacillus cereus* (Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979a), giving a product that appears to be the consequence of initial acylation of Ser-44 of this enzyme by the β-lactam (Knott-Hunziker et al., 1979b). Analogously, in the case of quinacillin sulfone (Fisher et al., 1980), the homologous Ser-45 (or the neighboring Thr-46) of the RTEM β-lactamase is labeled (J. Fisher and R. L. Charnas, unpublished experiments). Finally, Yocum et al. (1979) have shown that the serine residue of the D-Ala-D-Ala-carboxypeptidases from *Bacillus subtilis* and *Bacillus stearothermophilus* that is irreversibly acylated by the β-lactam of benzylpenicillin is part of a sequence that seems to show some homology with the β-lactamases. It is indeed tempting to suggest that the carboxypeptidases and the β-lactamases are all serine peptidases that proceed via acyl-enzyme intermediates, and that they only differ from each other in terms of substrate specificity and the relative rates of their acylation and deacylation steps. Only further structural and mechanistic studies can validate this postulate.

While the infrared spectral evidence suggests the existence of an essential serine, threonine, or tyrosine residue at the active site of the β-lactamases, these enzymes clearly do not resemble the serine proteases in every mechanistic respect. For instance, even though the β-lactam ring is cleaved first to generate an acyl-enzyme, this acyl-enzyme cannot be diverted to other products by the addition of surrogate nucleophiles. It is well known that acyl-enzymes from such proteases as α-chymotrypsin, trypsin, and elastase can readily be trapped by nucleophiles such as hydroxylamine, methanol, or amino acid esters (Inward & Jencks, 1965; Fastrez & Fersht, 1973). However, no product diversion can be observed during the enzymatic hydrolysis of either benzylpenicillin (k_{cat} = 2000 s^{-1}), or methicillin (k_{cat} = 10 s^{-1}) in the presence of [^{14}C]-methanol (R. L. Charnas, unpublished results), and whereas hydroxylamine is nearly 10^3-fold better than water in the deacylation of furoyl-α-chymotrypsin (Inward & Jencks, 1965), it does not affect the catalytic activity of the β-lactamase toward benzylpenicillin (Charnas et al., 1978) or cefoxitin (see Results). These findings must mean either that alternate nucleophiles such as methanol or hydroxylamine have no access to the susceptible link in the acyl-enzyme, or that the enzyme cannot exploit the inherently greater reactivity of such water analogues. On the other hand, hydroxylamine *can* partially reactivate β-lactamase that has been inactivated by either clavulanic acid (Charnas et al., 1978) or penicillanic acid sulfone (Fisher & Knowles, 1979) and can also participate in enzyme-catalyzed deacylation of penicilloyl-carboxypeptidases (Kozarich et al., 1977). The question of trapping acyl-β-lactamases must, therefore, remain open for the present.

The present experiments establish a strong mechanistic link between the D-Ala-D-Ala carboxypeptidases and the β-lactamases. It appears that both groups of enzymes are acylated on a unique serine residue by appropriate penams and cephems, but that only for the β-lactamases is hydrolytic *de*acylation an enzyme-catalyzed process. This mechanistic knowledge has already illuminated the problem of the rational design of inactivators of the β-lactamases (Fisher et al., 1980) and should give new momentum to the search for effective β-lactam antibiotics that are inert to (or can actually inactivate) the β-lactamases.

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Phosphorylated Intermediate of Alkaline Phosphatase†

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ABSTRACT: We have measured the phosphorylation of the subunits of alkaline phosphatase in the steady state with several substrates and at several pH values. Our results vary from 80% phosphorylation of *both* subunits at pH 7 to only 9% at pH 10. There is no evidence of anticooperativity. With the measurements of k_{cat}, we are able to evaluate rate constants in a minimal scheme. The results show that the main rate influencing steps are chemical dephosphorylation and dissociation of phosphate. The former predominates at pH 7.0 but declines in importance as the pH is raised. Our rate constants for dissociation of phosphate are in agreement with recent NMR studies.

Alkaline phosphates from *Escherichia coli* is a dimeric enzyme derived from a single cistron (Levinthal et al., 1962; Singer, 1961; Rothman & Byrne, 1963; Schlesinger & Levinthal, 1963; Levinthal et al., 1963). It is obtained as three major isozymes composed of three combinations of two slightly different subunits, A and B (Levinthal et al., 1962; Malamy & Horecker, 1964; Simpson et al., 1968; Lazdunski & Lazdunski, 1967; Singer et al., 1961; Schlesinger & Anderson, 1968; Levinthal et al., 1963). Isozyme I, the first to elute in chromatography from a DE-52 cellulose column has the composition A, A; isozyme II has the composition A, B; and isozyme III has B, B. Subunit B is derived from A by an epigenic modification, probably involving the removal of the N-terminal arginine amino acid residue (Kelley et al., 1973; Schlesinger et al., 1975). The enzyme binds 4 equiv of Zn^{2+} and 2 equiv of Mg^{2+} (Anderson et al., 1975).

The three isozymes can be displayed in polyacrylamide gel electrophoresis as the native enzyme and also as the apoenzyme, lacking Zn^{2+}, Mg^{2+}, and phosphate (McManaman & Wilson, 1978).

This enzyme follows Michaelis kinetics but shows substrate activation at high substrate concentrations, $\geq 10^{-3}$ M (Levinthal et al., 1962). This phenomenon has been the initial basis for the idea of anticooperativity between the subunits. Since then a number of papers have supported the idea of anitcooperativity. In this theory only one subunit is active at low substrate concentrations because the binding or reaction of substrate with the active site of one subunit greatly diminishes the affinity for substrate of the active site of the second subunit. This idea has been incorporated in the theory of a flip-flop mechanism (Lazdunski et al., 1971).

The reaction mechanism of this enzyme involves the formation of a covalent phosphoryl-enzyme intermediate (Morton, 1955; Agren et al., 1959; Engstrom & Agren, 1958; Engstrom, 1962; Schwartz & Lipmann, 1961; Barrett et al., 1969) analogous to the acetyl-enzyme intermediate of acetylcholinesterase and other enzymes that hydrolyze carboxylic esters (Wilson et al., 1950). The enzymic nucleophile is the hydroxyl group of a specific serine residue. Although kinetically labile, the phosphoryl-enzyme is amazingly stable (thermodynamically) as compared to other phosphate esters (Dayan & Wilson, 1963). Phosphate itself forms a very stable noncovalent complex with alkaline phosphatase, and the extra stability of the phosphoryl-enzyme can be attributed to the existence of noncovalent interactions similar to those that operate between phosphate and the enzyme in the noncovalent phosphate–enzyme complex.

The minimal scheme for the reaction mechanism is

$$\begin{array}{l} E + ROPO_3H_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot ROPO_3H_2 \xrightarrow{k_2} E\text{-}P + ROH \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad k_{-3} \Downarrow k_3 \pm H_2O \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad E\cdot P_i \xrightarrow{k_4} E + P_i \end{array} \qquad (1)$$

The step k_{-2} is omitted because [ROH] is almost zero in initial velocity measurements, and step k_{-4} is omitted for a similar reason. In this scheme, $E\cdot ROPO_3H_2$ represents the Michaelis–Mentin complex, E–P is the convalent phosphoryl-enzyme, $E\cdot P_i$ is the Michaelis–Menten complex between

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