Mechanism of Inhibition of the PC1 β-Lactamase of *Staphylococcus aureus* by Cephalosporins: Importance of the 3′-Leaving Group†

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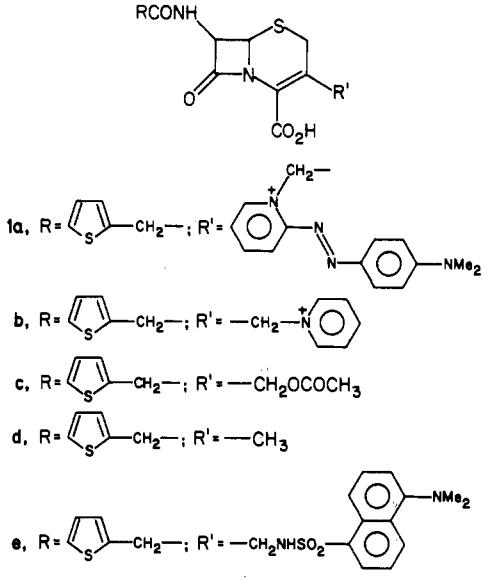
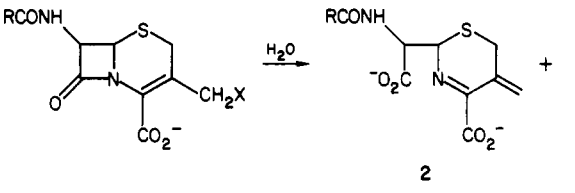
ABSTRACT: The hydrolysis of cephalosporins containing good leaving groups at the 3′-position [those used in this study were the chromogenic cephalosporin PADAC [pyridine-2-azo-4′-(*N*′,*N*′-dimethylaniline) substituted on cephalosporin], cephaloridine, and cephalothin], catalyzed by the *Staphylococcus aureus* PC1 β-lactamase, proceeds in two spectrophotometrically observable phases. The first involves formation of an acyl-enzyme intermediate while the second involves partitioning of this intermediate between two pathways. One path yields the normal cephalosporoate (**3**) from which the 3′-leaving group is spontaneously eliminated in solution to give the 3-methylenedihydrothiazine **2**, while the second involves initial elimination of the 3′ substituent, thus yielding a second acyl-enzyme intermediate, which then hydrolyzes to give the same final product as from the first pathway. The second acyl-enzyme is relatively inert to hydrolysis ($t_{1/2} \simeq 10$ min at 20 °C), and its formation thus leads to transient inhibition of the enzyme. The partition ratio between hydrolysis and elimination at the enzyme active site could be determined either spectrophotometrically from burst experiments or from measurements of residual β-lactamase activity as a function of cephalosporin concentration. This ratio varied with the leaving group ability of the 3′ substituent (acetoxy > *N*,*N*-dimethylaniline-4-azo-2′-pyridinium > pyridinium) in the anticipated fashion. The inert acyl-enzyme intermediate was isolated by exclusion chromatography and shown to contain the cephem nucleus, but not the 3′ substituent, covalently bound to the enzyme. As would be expected, PADAC, cephaloridine, and cephalothin yielded the same inert intermediate. Cephalosporins with poor or no 3′-leaving groups, e.g., dansylcephalothin and desacetoxycephalothin, neither displayed the branched pathway nor yielded the long-lived acyl-enzyme.

The cephalosporins (**1**) are currently widely used β-lactam



antibiotics and have, in general, supplanted penicillins in the treatment of stubborn infections. As would be anticipated in view of the clinical and commercial importance of these compounds, extensive structure/activity correlations have been made [see, for example, Gorman & Ryan (1972), Sassiver & Lewis (1977), Webber & Ott (1977), and Boyd (1982)]. The substituent in the 3-position has been shown to be particularly important in this regard since it modulates the electrophilicity of the β-lactam carbonyl group, which is an important parameter in the antibiotic activity of cephalosporins (Hermann, 1973; Indelicato et al., 1977; Boyd et al., 1980; Boyd, 1982). In most effective cephalosporin antibiotics the 3 substituent is an electron-withdrawing group, generally consisting of a suitable heteroatom functionality, attached either directly to the cephem ring or, more commonly, through a 3′-methylene group. The latter situation gives rise to further possibilities for activity modulation since it is known that elimination of good leaving groups from the 3′-position of a cephalosporin accompanies nucleophilic β-lactam ring cleavage, during hydrolysis, for example (eq 1) (Sabath et al., 1965; Newton et



$$2H^+ + X^- \quad (1)$$

al., 1968; Hamilton-Miller et al., 1970a,b). Since all clinically important reactions of β-lactam antibiotics involve such β-lactam cleavage (Ghuysen et al., 1979; Waxman & Strominger, 1982) it seems likely that the chronology of departure of such a leaving group could be an important factor in determining the effectiveness of the interactions of the relevant cephalosporins with β-lactam binding sites. This chronology has not yet been systematically investigated. Boyd has recently proposed, however, that the biological data do indicate that the presence of a leaving group at the 3′-position of a cephalosporin does produce a more effective antibiotic (Boyd, 1984).

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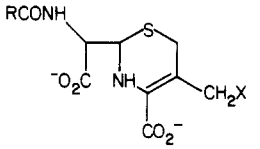
For many years it was believed, on both experimental and theoretical grounds, that elimination of a good 3′-leaving group was concerted with nucleophilic cleavage of the β-lactam ring (O'Callaghan et al., 1972; Waller, 1973; Bundgaard, 1975; Boyd et al., 1975; Boyd & Lunn, 1979; Boyd, 1982). Recently, however, we have shown that this synchrony need not occur and certainly does not when hydrolysis of the cephalosporins PADAC[1] (**1a**) and cephaloridine (**1b**) is catalyzed by the TEM-2 β-lactamase (Faraci & Pratt, 1984), where the β-lactam hydrolysis product (**3**) still bearing the 3′-leaving group



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is released by the enzyme into solution. The practical consequences of this result in terms of the details of the interactions of cephalosporins with β-lactamases and other β-lactam binding proteins are interesting and important. In some cases, like that of PADAC and the TEM-2 β-lactamase, the elimination will occur in solution. In other cases it might occur, either with or without enzyme participation, on the enzyme surface, subsequent to β-lactam cleavage. We present in this paper an example of the second kind, that of the PC1 β-lactamase of *S. aureus* with the substrates PADAC, cephaloridine, and cephalothin (**1c**), where elimination not only occurs at the enzyme active site but also yields thereby a transiently inhibited enzyme.

Experimental Procedures

Materials. The PC1 β-lactamase of *S. aureus* was obtained from the Centre for Applied Microbiology and Research, Porton Down, England, and used as supplied. It had a specific activity against benzylpenicillin of 140 units/mg at pH 7.5 and at 20 °C. Cephaloridine, cephalothin, and desacetoxycephalothin (**1d**) were generous gifts of Eli Lilly and Co., and PADAC was a generous gift of Hoechst-Roussel Pharmaceuticals Inc. All buffer materials were reagent grade.

Analytical Methods. All kinetics experiments were performed at 20 °C in 0.1 M potassium phosphate buffer at pH 7.5, unless otherwise stated. Absorption spectra and steady-state reaction rates were measured by means of a Cary 219 spectrophotometer. β-Lactamase activity was routinely estimated against benzylpenicillin by the spectrophotometric method of Waley (1974). Pre-steady-state kinetics measurements were obtained from a Durrum D-110 spectrophotometer (Anderson & Pratt, 1981, 1983). The β-lactamase concentrations were determined spectrophotometrically, assuming 1.99×10^4 M^{-1} cm^{-1} was the extinction coefficient of the protein at 276.5 nm (Carrey & Pain, 1978).

Steady-State Kinetics. Michaelis–Menten parameters were obtained by the method of Wilkinson (1961) from initial velocity measurements of the hydrolysis of the various cephalosporins. With substrates where a good leaving group was present at the 3′-position, these velocities are of course those measured for the steady state subsequent to the burst described below. The reactions were followed spectrophotometrically at 570 nm for PADAC and at 260 nm for cephaloridine, cephalothin, and desacetoxycephalothin. Enzyme concentrations were 0.1 μM and substrate concentrations between 1 and 10 μM. The extinction coefficient of PADAC at 570 nm was taken as 57 000 M^{-1} cm^{-1} (Schindler & Huber, 1980).

Pre-Steady-State Kinetics. The reaction of PADAC (4.8–14.0 μM) with the PC1 β-lactamase (4.8 μM) was followed spectrophotometrically at 570, 498, and 260 nm in the stopped-flow instrument. The resulting absorption changes were fitted to reaction schemes by the nonlinear least-squares procedures described previously (Anderson & Pratt, 1981, 1983).

Observation and Measurement of the Pre-Steady-State Burst on Reaction of the PC1 β-Lactamase with PADAC. The burst size was measured as the rapid (complete in 10 s) absorbance drop at 570 nm on addition of small aliquots of the enzyme (final concentration 1.3 μM) to solutions (final volume 1.0 mL) of PADAC (final concentrations between 7.0 and 31.0 μM).

Measurement of the Extent of Inhibition of the Enzyme Produced by Cephalosporins. An aliquot of enzyme, to a final concentration of 3.0 μM, was added to a solution of the cephalosporin (final volume 0.3 mL; final concentrations 0.5–7.0, 1.0–35.0, 0.5–3.5, and 67.0 μM for PADAC, cephaloridine, cephalothin, and desacetoxycephalothin, respectively). An aliquot of the mixture was withdrawn after 20 s and added to the standard benzylpenicillin assay mixture. The initial velocity of benzylpenicillin hydrolysis was taken as a measure of the amount of immediately active enzyme remaining. The absorbance of the benzylpenicillin was also followed over longer times in order to determine the rate of return of β-lactamase activity.

Isolation of an Acyl-Enzyme. A 60-μL aliquot of PADAC solution (3.55 mM) was added to 0.90 mL of a solution at room temperature containing 100 μM β-lactamase. After about 30 s this mixture was loaded onto a Bio-Gel P-4 column (1.0 × 22 cm) at 4 °C and eluted at a flow rate of 1.5 mL/min with 20 mM phosphate buffer at pH 7.5 and at the same temperature. Fractions of 0.5 mL were collected and those containing enzyme identified through their absorbance at 276.5 nm.

Assay for the Methylenedihydrothiazine (**2**) *and Cephalosporins Giving Rise to It.* A quantitative spectrophotometric assay for **2** and those cephalosporins which give rise to it on β-lactam ring cleavage, i.e., those with 3′-leaving groups, was devised and is now described and may be of general interest. Concentrated sodium hydroxide solution was added to solutions of **2** or of an appropriate cephalosporin, to a final hydroxide concentration of 0.8 M. After 4 h an absorption peak at 330 nm ($\epsilon = 6100$ M^{-1} cm^{-1}) was completely formed and remained unchanged for at least 2 days. The absorbance was linear with the concentration of **2** up to 120 μM at least. The NMR spectrum and the absorption spectrum of the chromophoric product were identical with that of the product of alkaline hydrolysis of benzyl 4-carboxylato-5-methyl-6*H*-1,3-thiazin-2-yl-2′-thienylacetamidoacetate (**4**), which is most likely **5**. The benzyl ester of **5** was prepared by reaction of cephalothin with sodium benzyl oxide as described by Eggers et al. (1965) and after recrystallization from ethyl acetate had the following properties: mp 194–195 °C; ^1H NMR ($[^2H_6]Me_2SO$) δ 2.24 (s, 3, CH_3), 3.43 (s, 2, 2-H), 3.74 (s, 2, $ThCH_2$), 5.13 (s, 2, $PhCH_2$), 6.94 (d, $J = 4$ Hz, 2, Th3′,5′), 7.3–7.5 (m, 6, Ph, Th4′), 9.07 (s, 1, CONH), 11.28 (s, 1, OH or NH); UV (H_2O) λ_{max} 340 nm ($\epsilon = 17\,200$ M^{-1} cm^{-1}). Satisfactory C, H, and N analyses for $C_{21}H_{20}N_2S_2O_5$ were obtained. These reactions are shown in Scheme I.

[1] Abbreviations: PADAC, pyridine-2-azo-4′-(*N′,N′*-dimethylaniline) substituted on cephalosporin [(6*R-trans*)-1-[[2-carboxy-8-oxo-7-[(2-thienylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]-2-[[4-(dimethylamino)phenyl]azo]pyridinium hydroxide, inner salt]; Me_2SO, dimethyl sulfoxide.

Scheme I

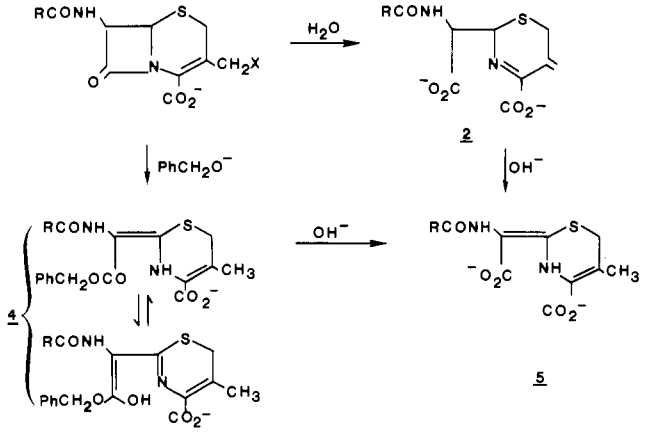


Table I: Steady-State Parameters for Hydrolysis of Cephalosporins Catalyzed by the PC1 β-Lactamase

substrate	k_{cat} (s^{-1})	K_m (μM)
PADAC	$(2.2 \pm 0.1) \times 10^{-3}$	<0.5[a]
cephaloridine	$(8.0 \pm 1.0) \times 10^{-3}$	<1[a]
desacetoxycephalothin	1.0 ± 0.05	15.4 ± 2.1
dansylcephalothin	0.64 ± 0.1	1.7 ± 0.5

[a] An upper limit established by the lower limit of substrate concentrations which could allow the reaction to be followed spectrophotometrically.

^1H NMR Spectra of Reaction Products. Small portions of solid enzyme were added to NMR tubes containing the cephalosporin substrate (10 mM) and sodium bicarbonate (20 mM) in ^2H_2O. NMR spectra were then recorded at intervals until the enzyme-catalyzed reaction was complete.

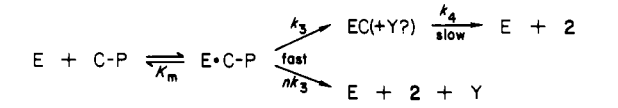
Results

^1H NMR spectra showed that the PC1 β-lactamase-catalyzed hydrolysis of PADAC and cephaloridine yielded the corresponding pyridine and **2**. These are the products expected on the basis of all previous work, most notably by Abraham and co-workers (Newton et al., 1967, Hamilton-Miller et al., 1970a). The NMR spectrum of **2** observed here [^2H_2O, δ 3.40, 3.70 (ABq, J = 15 Hz, 2, 2-H), 3.86, 3.94 (ABq, J = 16 Hz, 2, $ThCH_2$), 4.65 (d, J = 3 Hz, 1, H-7), 5.45 (d, J = 3 Hz, 1, H-6), 5.63 (s, 1, =CH), 5.68 (s, 1, =CH), 7.03 (m, 2, Th3′,5′), 7.38 (m, 1, Th4′)] is in accord with spectra recorded by Hamilton-Miller et al. (1970b). Hence, β-lactamase-catalyzed hydrolysis of these cephalosporins leads to elimination of the 3′-leaving group. The NMR spectrum of the β-lactamase-catalyzed hydrolysis product of desacetoxycephalothin [^2H_2O, δ 1.86 (s, 3, CH_3), 3.10, 3.42 (ABq, 2, J = 17 Hz, 2-H), 3.91 (s, 2, $ThCH_2$), 4.54, 4.61 (ABq, J = 5 Hz, H-6, H-7), 7.06 (m, 2, Th3′,5′), 7.38 (m, 1, Th4′)] is suggestive of structure **3** where no rearrangement has accompanied β-lactam cleavage; this too is in accord with expectation and previous observation (Hamilton-Miller et al., 1970b).

Steady-state parameters for the hydrolysis of these cephalosporins, along with those for dansylcephalothin (**1e**) obtained under the same conditions, are given in Table I. Previous work has shown that the dansylamido group does not leave on hydrolysis of dansylcephalothin in the presence of the PC1 β-lactamase (Anderson & Pratt, 1981). The low k_{cat} and K_m of PADAC and cephaloridine, where a leaving group is present at C-3′, are suggestive of the accumulation of a quite stable intermediate. This idea is demonstrated to be true below.

Addition of enzyme to solutions containing saturating PADAC concentrations leads to a burst of substrate disappearance and of *N,N*-dimethylaniline-4-azo-2′-pyridine formation. This

Scheme II



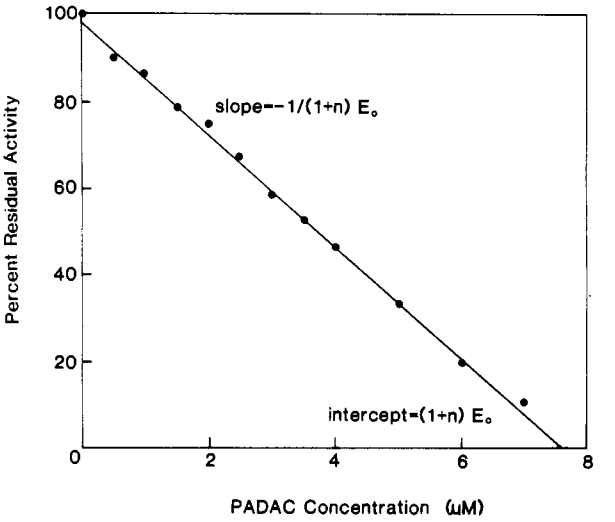


FIGURE 1: Residual immediate β-lactamase activity against benzylpenicillin after incubation of the enzyme (3.0 μM) with various concentrations of PADAC. The points are experimental, and the line is calculated as described in the text.

was monitored, as described above, at 570 nm (the disappearance of PADAC, or, more precisely, the disappearance of the *N,N*-dimethylaniline-4-azo-2′-pyridinium group) or at 470 nm (appearance of *N,N*-dimethylaniline-4-azo-2′-pyridine). The burst, which is too fast to be followed at the concentrations employed by manual mixing methods, is succeeded by the steady-state rate reported above. The stoichiometry of the burst, π, was determined to be (1.88 ± 0.10) times the enzyme concentration. A burst of magnitude greater than the enzyme concentration indicates that a branched pathway must exist where one branch regenerates the free enzyme necessary to explain the multiple turnover. Accordingly, the minimal reaction sequence of Scheme II can be set up where C–P represents PADAC which is composed of the cephem nucleus C and the purple leaving group P which becomes yellow, Y, after elimination from C. In terms of this equation the accumulation of enzyme as EC gives rise to the burst, and the slow breakdown of EC is largely responsible for the steady-state rate. Significant uncertainties to be explored are the nature of C–P in E·C–P and in EC. The burst stoichiometry π/E_0 will be equal to either $n + 1$ or n depending on whether Y is released on formation of EC or not.

The partition ratio of E·C–P, n, can be obtained through measurements of activity remaining immediately after incubation of the enzyme with small concentrations of PADAC. If it is assumed that E·C–P dissociates rapidly, the instantaneous enzyme activity, A, measured against benzylpenicillin, is given by eq 2 where A_0 is the initial activity of the enzyme

$$A/A_0 = (E + \text{E·C–P})/E_0 = 1 - (\text{EC}/E_0) = 1 - S_0/[(1 + n)E_0] \quad (2)$$

at a total concentration E_0 and A the measured activity immediately (20 s) after addition of PADAC at a final concentration S_0 [where $S_0 < (1 + n)E_0$; at $S_0 \geq (1 + n)E_0$, A is zero of course). Figure 1 shows a plot of A/A_0 vs. S_0 for PADAC which yields a straight line, as required by eq 2, and thence an n value of 1.2. Since the value of π is closer to that of $n + 1$ than of n, Y is probably formed with EC. Further

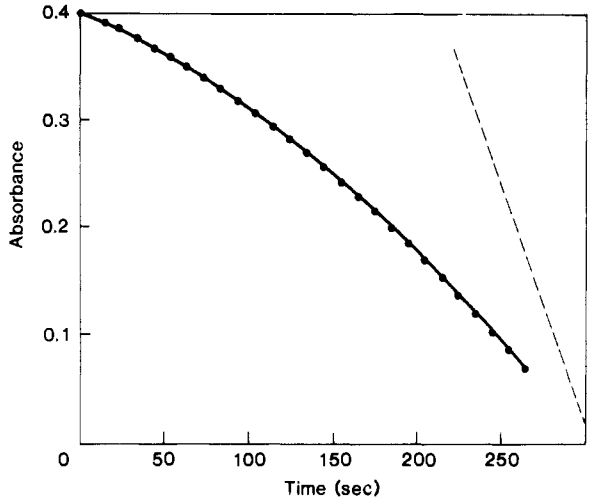


FIGURE 2: Return of β-lactamase activity on dilution of an enzyme (3.0 μM)/PADAC (6.0 μM) mixture into the benzylpenicillin assay mixture. The points are experimental, and the solid line is calculated as described in the text. The dashed line represents the initial activity or that at long times after dilution.

evidence for this conclusion is given below.

On immediate dilution of the above enzyme/PADAC mixtures [with PADAC concentrations $\leq (1 + n)E_0$] into a benzylpenicillin assay medium, the enzyme activity, initially corresponding to E plus E·C–P, increased with time as EC broke down regenerating E. These curves can be analyzed by using the eq 3–6, below, where B is benzylpenicillin and Q is

$$EC \xrightarrow{k_4} E + 2 \quad (3)$$

$$E + B \rightleftharpoons EB \xrightarrow{k_{cat}^A} E + Q \quad (4)$$

$$\frac{dQ}{dt} = k_{cat}^A E \quad (5)$$

$$\frac{dE}{dt} = k_4 EC \quad (6)$$

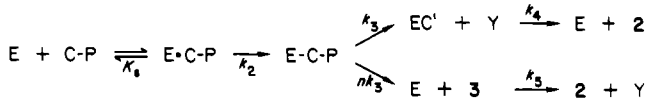
benzylpenicilloate and where, initially, E is equal to the E + E·C–P of Scheme I. Under conditions of $B_0 \gg K_m^A$, E_0, eq 5 and 6 apply. An example of one of these curves for PADAC is given in Figure 2. Numerical analysis using the previously described nonlinear least-squares routine (Anderson & Pratt, 1981, 1983) yielded the only unknown, k_4, as 0.0012 s^{-1}. The good fit of the calculated curve to the experimental data is also indicated in Figure 2. Scheme II also leads to eq 7. It

$$k_{cat} = (1 + n)k_4 \quad (7)$$

is clear that the measured values of k_4 and n predict the k_{cat} of PADAC (Table I) very well.

The influence of the 3′ substituent on the above parameters was investigated by using the substrates cephalothin (3′-acetoxy), cephaloridine (3′-pyridinium), and desacetoxycephalothin (3-H). Experiments of the type described above yielded $n = 11$ and $k_4 = 0.0010$ for cephaloridine and $n = 0$ and $k_4 = 0.0010$ for cephalothin. In passing, it might be noted that $(1 + n)k_4$ for cephaloridine also gives a good approximation to its k_{cat} value (Table I). These results are quite significant. The identity of k_4 for the three substrates, PADAC, cephaloridine, and cephalothin, suggests that EC is a common intermediate, which provides further evidence that Y (or the corresponding leaving group) has been eliminated from EC. The order of n values can be compared with the order of leaving group abilities of the 3′-substituents as determined by the rates of elimination of these leaving groups from 3. Rate constants for the spontaneous elimination of X = pyridine and X = dimethylaniline-4-azo-2′-pyridine from 3 under the same conditions as employed here are 0.44 s^{-1} and 11 s^{-1}, respectively (Faraci & Pratt, 1984). The rate constant for acetoxy elimination has not been yet determined, but a preliminary experiment shows that it is certainly greater than the above numbers. It thus appears that to a good approximation, n is inversely proportional to the rate of elimination of X from 3, suggesting that the formation of EC involves elimination of X and further, therefore, that the direct breakdown of E·C–P to E does not. Desacetoxycephalothin (up to 67 μM, equivalent to $20E_0$) gave rise to no transient loss of β-lactamase activity. This suggests that an intermediate corresponding to EC does not accumulate in this case, which is perfectly understandable if elimination of a 3′ substituent is an essential part of its formation. Transient phenomena of this type are not seen in the interaction of dansylcephalothin with the PC1 β-lactamase either (Anderson & Pratt, 1981, 1983), where it has been proved that no elimination occurs.

Scheme III



Stopped-Flow Studies with PADAC. These were undertaken to gain further insight into the nature of the intermediates. Typical data at a particular PADAC concentration are shown in Figure 3. These curves indicate the time course of events preceding the steady state. Two steps are clearly evident, indicating the presence of an intermediate which cannot be EC in view of its rate of decay. Taking into account the results and their interpretation presented above, the known behavior of 3 (Faraci & Pratt, 1984), and the current state of knowledge of β-lactamase mechanisms (Anderson & Pratt, 1981, 1983; Knowles, 1983; Cartwright & Waley, 1983), these data were quantitatively analyzed according to expanded Scheme III. The transient intermediate referred to above is thus E–C–P, which is most likely an acyl-enzyme, since its formation is accompanied by a decrease in absorbance at 260 nm (Figure 3) which is characteristic of β-lactam cleavage (Anderson & Pratt, 1981). Since E–C–P is an acyl-enzyme there must presumably be a noncovalent intermediate (E·C–P) preceding it, hence, its inclusion in the scheme. The value of k_5 (11 s^{-1}) for the spontaneous elimination reaction of 3 in solution is available from the previous study (Faraci & Pratt, 1984). Quantitative fitting of the data to Scheme III by the simplex optimized nonlinear least-squares routine (Anderson & Pratt, 1981, 1983) was carried out with the further assumptions that the extinction coefficients at all three wavelengths of E·C–P was the same as that of E plus C-P and that the extinction coefficient of E–C–P was the same as that of E plus 3 (the justification for the latter assumption becomes clear below). The data were fitted with K_s, k_2, k_3, and the extinction coefficient of E–C–P as variable parameters (values of n were taken as 1.0 for most of these calculations, and k_4 was calculated from k_{cat} and n via eq 7). The mean values of the parameters and their standard deviations, from the optimized fits at all the PADAC concentrations employed, are given in Table II.

Of the extinction coefficients of E–C–P required to fit the data, those at 260 and 570 nm are the most informative. The extinction coefficient change at 260 nm between E plus C–P and E–C–P was found to be −3600 M^{-1} cm^{-1}. This is in good agreement with that observed on formation of the acyl-enzyme intermediate between the PC1 β-lactamase and dansylcep-

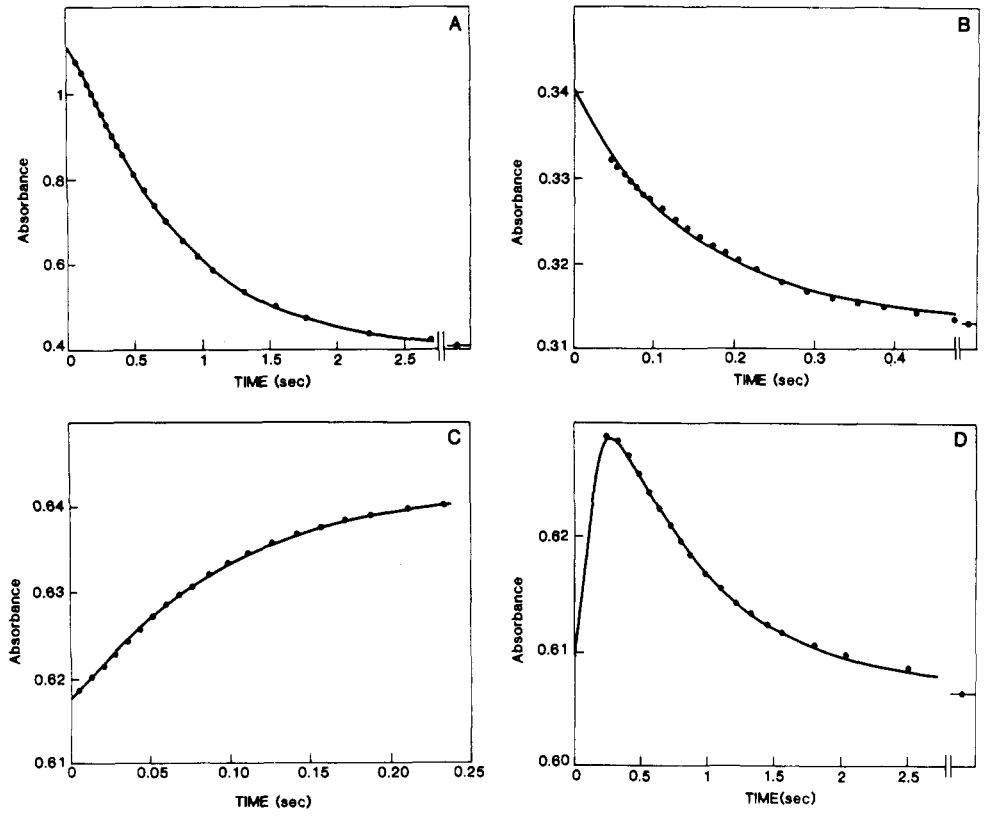


FIGURE 3: Absorbance changes at 570 (A), 260 (B) and 498 nm (C, D) on mixing β-lactamase (4.9 μM) with PADAC (14.0 μM) in the stopped-flow spectrophotometer. The points are experimental and the lines calculated as described in the text.

Table II: Rate Parameters for Hydrolysis of Cephalosporins Catalyzed by the PC1 β-Lactamase

substrate	K_s (μM)	k_2 (s^{-1})	k_3 (s^{-1})	k_3'[c] (s^{-1})	k_4 (s^{-1})	k_5[b] (s^{-1})
PADAC	10.5 ± 3.5	16.5 ± 2.5	1.8 ± 0.3	1.8 ± 0.3	0.0011 ± 0.0001	11
cephaloridine	*a*	*a*	0.6	0.055	0.0010	0.44
cephalothin	*a*	*a*	*a*	$\geq 10k_3$	0.0010	≥50
dansylcephalothin[d]	7.6 ± 0.3	5.6 ± 0.7	1.2 ± 0.2	0		0

[a] Not determined. [b] Faraci & Pratt (1984). [c] Calculated from $k_3' = k_3/n$. [d] Anderson & Pratt (1983).

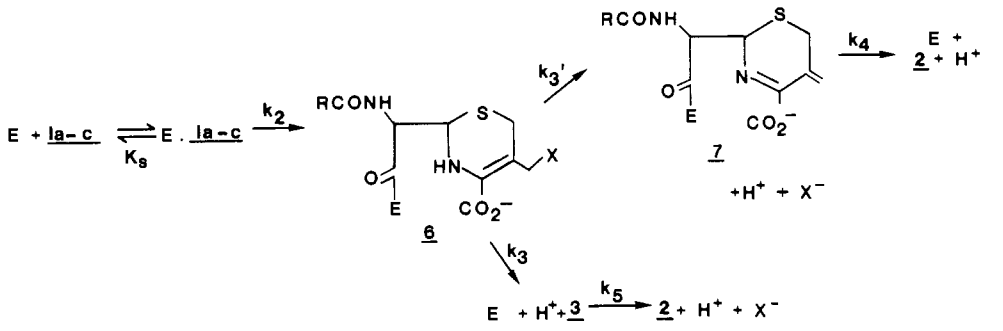
halothin where the change was −4000 M^{-1} cm^{-1} (Anderson & Pratt, 1981) and with those on hydrolysis of desacetoxycephalothin (−4200 M^{-1} cm^{-1}) and cephalexin (−3400 M^{-1} cm^{-1}). A similar change at 260 nm (−3500 M^{-1} cm^{-1}) was observed on conversion of PADAC into **3** by the TEM-2 β-lactamase. This provides good evidence for the opening of the β-lactam ring on formation of E–C–P and thus for it being a covalent acyl-enzyme. The extinction coefficient of E–C–P at 570 nm was determined to be 47 000 M^{-1} cm^{-1}. This compares very nicely with that of **3** (X = *N*,*N*-dimethylaniline-4-azo-2′-pyridine) at this wavelength, determined in earlier work (Faraci & Pratt, 1984) to be 46 000 M^{-1} cm^{-1}. This tends to confirm the presence of the 3′-dimethylaniline-4-azo-2′-pyridinium substituent in E–C–P.

The fit of the model of Scheme III to the stopped-flow data is seen in Figure 3 to be good, and thus, these data too are in accord with the emerging picture of events. Similar experiments were carried out with cephaloridine yielding the k_3 value given in Table II; since high cephaloridine concentrations were used in these experiments, K_s and k_2 were not well defined. As in the case of PADAC, *n* values for these calculations were taken from the residual activity experiment described above.

Identification of EC′ as a Covalent Complex. An important remaining uncertainty is that of the nature of EC′ (Scheme III). The leaving group has probably been eliminated at this stage but C′ may remain covalently bound to the enzyme. The alternative is that C′ has also been released and EC′ is actually E′, a conformationally modified enzyme, which only slowly reverts to E. EC′ was isolated as described under Experimental Procedures. Visual inspection, confirmed by an absorption spectrum, showed that the chromophoric 3′ substituent was indeed not present. The initial β-lactamase activity of the material was 10% of the original, which, taken with the analytical data given below, indicates that EC′ is inactive. The absorption spectrum of EC′ showed an apparently unchanged protein absorption above 270 nm but increased absorbance at lower wavelength. The spectrum also changed with time, in the form of a decrease in absorbance between 230 and 270 nm. This change was quantitatively analyzed at 20 °C and found to be a first-order decay with rate constant 0.0010 s^{-1}. The spectral change thus correlates with the return of activity, and indeed the activity of the isolated EC′ had returned to that of the native enzyme after completion of the spectral change.

Sodium hydroxide solution, to a final concentration of 0.8 M, was added to a portion of the EC′ solution that had aged at room temperature for 4 h. This led to the appearance of an absorption peak at 330 nm at the same rate as observed with standard solutions of **2** (prepared from hydrolyzed cephalothin; see Experimental Procedures for details of this assay). After the chromophore formation was complete, measurement of peak intensities gave the concentration of **2**

Scheme IV



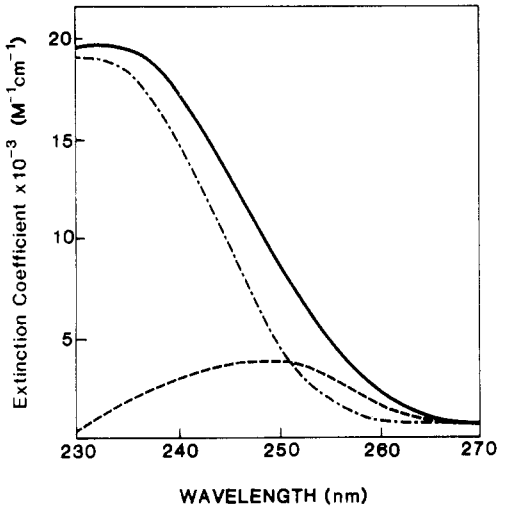


FIGURE 4: Difference spectrum (vs. free enzyme) of the covalent EC′ complex 7 (—) obtained as described in the text. Also shown are the difference spectrum between 7 as initially isolated and that after complete reaction (- - -) and the absorption spectrum of 2 (· · ·).

as 0.90 times that of enzyme. Thus, EC′ probably retains one equivalent of the cephalosporin nucleus covalently bound to it. Passage of another portion of the aged and reactivated EC′ solution through the P-4 column yielded protein fractions which now did not contain **2**. Thus, recovery of active enzyme from EC′ involves loss of the covalently bound (but 3′-substituent free) cephalosporin nucleus. This result is obviously in accord with the observation of significant spectral changes accompanying this process.

A difference spectrum generated on aging of EC′, i.e., on its conversion to E plus **2**, is shown in Figure 4. Also shown is the spectrum of **2**. The sum of these should give the difference spectrum of EC′ vs. the native enzyme, and this too is shown in Figure 4. It is clear that the enzyme-bound chromophore in EC′ strongly resembles the chromophore of **2**.

Similar experiments were performed with cephaloridine. EC′ was isolated and shown to generate 0.85 mol of **2**/mol of enzyme on hydrolysis; the decomposition of EC′, followed spectrally at 20 °C, also occurred with a rate constant of 0.0010 s^{-1}, confirming again the common identity of EC′.

Discussion

Previously described experiments in this laboratory (Anderson & Pratt, 1981, 1983) have demonstrated that the hydrolysis of cephalosporins, catalyzed by the PC1 β-lactamase of *S. aureus*, can be described by a three-step mechanism which includes a covalent acyl-enzyme intermediate, E–S (eq 8). The results of the current investigation, described above,

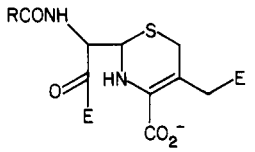
$$E + S \underset{K_s}{\rightleftharpoons} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{–}S \xrightarrow{k_3} E + P \qquad (8)$$

show that if a good leaving group is present at the 3′-position of the cephalosporin (which was not true in the earlier study which employed dansylcephalothin), elimination of that leaving group can occur at the acyl-enzyme stage. The experiments described show that the hydrolysis of PADAC, cephaloridine, and cephalothin in the presence of the PC1 β-lactamase follows the paths of Scheme IV. The acyl-enzyme (**6**) derived from these substrates, partitions between direct hydrolysis, which leads to complete turnover yielding **3**, and thus spontaneously **2**, and elimination, leading to a second acyl-enzyme (**7**), which is slower to hydrolyze than **6** and whose formation thus results in a decrease in turnover rate with time, as the enzyme funnels into **7**. The half-life of **7**, it should be noted, is around 10 min at 20 °C.

The partitioning, $n = k_3/k_3'$, of **6** appears to be strongly influenced by the leaving group ability of the 3′ substituent (Table II); the value of k_3' appears to be leaving group dependent while that of k_3 is not. The order of leaving group abilities (acetate > *N*,*N*-dimethylaniline-4-azo-2′-pyridine > pyridine) agrees with that found earlier (Faraci & Pratt, 1984) for the elimination reaction of **3** in free solution and is indicated by k_5 in Table II. A complete spectrum of leaving group chronology has thus emerged. With very good leaving groups (e.g., acetate), k_3' is much greater than k_3, and the enzyme is trapped as **7** on interaction with a single substrate molecule. In this case all turnover passes through **7**, and $k_{cat} = k_4$. With poorer leaving groups (e.g., the above-mentioned pyridines) significant normal turnover occurs prior to trapping. In the steady state, turnover will proceed via both **7** and **3**, and k_{cat} will be greater than k_4. In each of these cases, depending on the leaving group and on the enzyme/substrate concentration ratio, all of the substrate could be consumed before significant concentrations of **7** arose. Finally, with very poor or nonleaving groups at the 3′-position, where $k_3 \gg k_3'$, such as with the dansylamido group of **1e** or the hydrogen of **1d**, no elimination occurs at the active site, and **7** does not form.

Of considerable interest and importance, clearly, is the fact that **7** is considerably, by a factor of around 10^3, more inert to hydrolysis than **6**, since this leads to a slowly reversible covalent inhibition of the enzyme. This would be in accord with early observations that cephalothin and cephaloridine were effective antibiotics against β-lactamase-producing *S. aureus* (Wick, 1972). As Charnas & Knowles (1981) have pointed out, with reference to β-lactamases, totally irreversible inactivation of an enzyme is not essential for pharmaceutical efficacy. The reason for the inert nature of **7** must lie with its structure. Although, in the virtual absence of any information on the structure of β-lactamase active sites, firm conclusions are not possible, certain alternatives with respect to the substrate moiety can be considered. Firstly, it seems unlikely that the gross size and shape of the substrate in **7** lead to its resistance to hydrolysis. The k_{cat} value for desacetoxycephalothin (Table I), where the 3 substituent is of similar

size to that in **7**, is comparable to the values of k_3 for the substrates with larger substituents (Table II), and thus, k_3 for desacetoxycephalothin must be at least as great.



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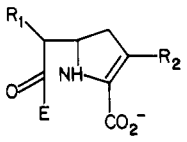
An attractive possibility, on paper at least, is that the structure of **7** is actually **8**, where the acyl-enzyme has been putatively stabilized through Michael addition of a protein functional group to the exomethylene group of **7**, thus cross-linking the enzyme. This does not appear to be correct either, since the absorption spectrum of the substrate moiety in EC′ (Figure 4) is much more suggestive of that of the methylenedihydrothiazine structure of **2** than of the dihydrothiazine of **3**. Exploratory experiments with ethylenediamine, mercaptoethanol, and imidazole (0.1 M each) in solutions of **2** gave no indication that the exomethylene group of **2** was susceptible to addition of nucleophiles at neutral pH; i.e., if there is an equilibrium in solution between **2** and **3**, it lies far toward **2** with these nucleophiles.

Finally, and more speculatively, one might suggest that the important difference between **6** and **7** is the absence of an N–H hydrogen bond donor in the latter. This might be necessary to maintain, or, to productively change, the enzyme conformation at this stage of catalysis. Conversely, the occurrence of an H bond acceptor in **7**, coplanar with the dihydrothiazine ring, may lead to an unwanted conformational change. There are certainly indications from the effect of antibodies on the cephaloridine-treated enzyme that conformational effects are involved in the inactivation produced by this reagent (Carrey et al., 1984). At this point it is also interesting to note that Knowles and co-workers (Charnas & Knowles, 1981; Easton & Knowles, 1982) have proposed that inactivation of the TEM β-lactamase by olivanic acids and related carbapenems arises from rearrangement of the normal acyl-enzyme intermediate, which contains a Δ^2-pyrroline moiety (**9**), into a Δ^1-pyrroline form (**10**). Possibly the same mechanism of stabilization of



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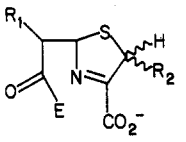

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the acyl-enzyme is involved and which thus may be of general significance.

In broader perspective, the mechanism of Scheme IV leading to the transient inhibition of the PC1 β-lactamase by cephalosporins is typical of that observed with what is now an extensive range of mechanism-based β-lactamase inhibitors [reviewed, for example, by Sykes & Bush (1982), Knowles (1983), and Cartwright & Waley (1983)], all of which involve the partitioning of an acyl-enzyme intermediate into a more hydrolytically inert form. In many cases, such as those of the 6-β-halopenicillanic acids, the penam sulfones, and clavulanic acid, a chemical rearrangement of the substrate occurs, after β-lactam ring opening, which, in itself, in view of the structure of the rearranged substrate, may account for the increased stability. In other cases, such as the carbapenems and now the cephalosporins, it seems that although a chemical rearrangement does occur, the stability must arise through a change in enzyme structure, since the chemical change to the substrate itself would not seem to lead to any change in the intrinsic chemical reactivity of the acyl-enzyme toward hydrolysis. Finally, there are examples, such as the sterically hindered penicillins, methicillin, etc., where only an enzyme conformational change appears to bring about stabilization of the intermediate.[2] Whether the β-lactamase active site is generally more susceptible to inactivation by distortion than those of other enzymes or whether it is just more subject to the variations of a versatile substrate under strong selective pressure is not known at present. Certainly the Gram-positive β-lactamases, including the PC1 β-lactamase, give evidence of significant conformational flexibility (Citri & Pollock, 1966; Citri et al., 1976; Pain & Virden, 1979).

Of clear and immediate interest is the extent to which the mechanism of Scheme IV is relevant to other β-lactamases and to other β-lactam binding proteins. The presence of the partitioning of **6** would be dictated by the relative values of k_3 which can presumably be influenced by the substituents at C-7 and of k_3' which, as shown in this work, is a strong function of the leaving group ability of the 3′ substituent. It is pertinent to point out here that the order of leaving group abilities (as opposed to electron-withdrawing power) of substituents of different chemical structure is not easily predictable either by experiment (Stirling, 1979) or yet by theory (Boyd, 1982). The enzyme itself will clearly be an important further contributor. Comparison of the available values of k_3' and k_5 (Table II) suggests that the PC1 β-lactamase does not greatly affect the rates of elimination. Other enzymes may influence the situation differently. We have already noted for example (Faraci & Pratt, 1984) that *Bacillus cereus* β-lactamase II catalyzes departure of the 3′-leaving group of PADAC from the enzyme surface. We are currently investigating the range of these possibilities.

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[2] It might be noted here that the inactivation of the PC1 β-lactamase by cephaloridine was previously grouped with others of this type (Virden et al., 1978; Pain & Virden, 1979; Carrey et al., 1984). It should also be mentioned, however, that an explanation similar to that given in this paper was previously suggested by Dr. A. F. W. Coulson and presented at the β-Lactamase Workshop, Newcastle-upon-Tyne, April 27–29, 1979.

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Association–Dissociation Modulation of Enzyme Activity: Case of Lactose Synthase†

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ABSTRACT: Lactose synthase was found to show anomeric preference for β-D-glucose. This information was utilized in the design of methyl, ethyl, propyl, butyl, and pentyl *N*-acetyl-β-D-glucosaminides, which were subsequently demonstrated to be substrates for galactosyltransferase with apparent K_m values in the low millimolar range. α-Lactalbumin competitively inhibits the transferase activity against these *N*-acetylglucosamine derivatives. This pattern of inhibition has also been observed when the dimer, trimer, and tetramer of *N*-acetylglucosamine and ovomucoid served as the galactose acceptor. The data suggest that the binding of α-lactalbumin and the *N*-acetylglucosamine derivatives is mutually exclusive. This assertion is further supported by the inability of methyl and butyl *N*-acetyl-β-D-glucosaminides to facilitate retention of galactosyltransferase on a column of α-lactalbumin immobilized onto Sepharose. Free *N*-acetylglucosamine, on the other hand, does cause retention of the transferase under the same conditions. Thus, α-lactalbumin must bind to a region on galactosyltransferase in close proximity to the monosaccharide binding site and exert its substrate-specifying action through competitive and mutually exclusive binding with the *N*-acetylglucosamine analogues accompanied by an increased affinity for glucose. In short, our substrate analogue studies have revealed that the association–dissociation modulation of galactosyltransferase activity is effected through a topographical blockade of glycoprotein binding by α-lactalbumin.

Association–dissociation modulation of enzymatic activity represents another form of enzyme regulation. In this process, the activity of an enzyme is increased or decreased or its substrate specificity modified upon association or dissociation of its heterosubunits. The activation of cAMP-dependent protein kinase in the presence of cAMP is an example of inhibition through association of heterosubunits (Gill & Garren, 1970; Brostrom et al., 1970). On the other hand, the

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