Mechanism of Inhibition of RTEM-2 β-Lactamase by Cephamycins: Relative Importance of the 7α-Methoxy Group and the 3′ Leaving Group†

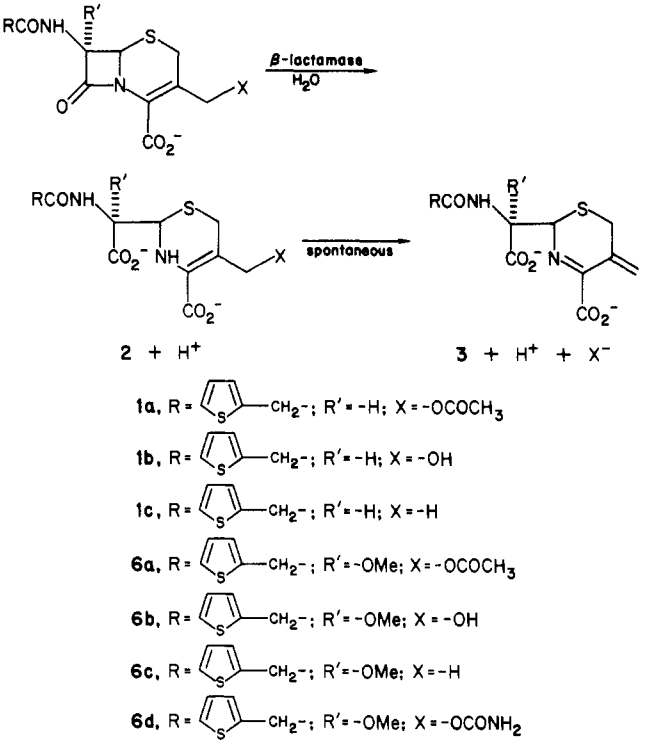
W. Stephen Faraci and R. F. Pratt*
Department of Chemistry, Wesleyan University, Middletown, Connecticut 06457
Received September 4, 1985; Revised Manuscript Received January 14, 1986

ABSTRACT: Cefoxitin is a poor substrate of many β-lactamases, including the RTEM-2 enzyme. Fisher and co-workers [Fisher, J., Belasco, J. G., Khosla, S., & Knowles, J. R. (1980) *Biochemistry 19*, 2895–2901] showed that the reaction between cefoxitin and RTEM-2 β-lactamase yielded a moderately stable acyl-enzyme whose hydrolysis was rate-determining to turnover at saturation. The present work shows first that the covalently bound substrate in this acyl-enzyme has a 5-*exo*-methylene-1,3-thiazine structure, i.e., that the good (carbamoyloxy) 3′ leaving group of cefoxitin has been eliminated in formation of the acyl-enzyme. Such an elimination has recently been shown in another case to yield an acyl-β-lactamase inert to hydrolysis [Faraci, W. S., & Pratt, R. F. (1985) *Biochemistry 24*, 903–910]. Thus the cefoxitin molecule has two potential sources of β-lactamase resistance, the 7α-methoxy group and the good 3′ leaving group. That the latter is important in the present example is shown by the fact that with analogous substrates where no elimination occurs at the enzyme active site, such as 3′-de(carbamoyloxy)cefoxitin and 3′-decarbamoylcefoxitin, no inert acyl-enzyme accumulates. An analysis of the relevant rate constants shows that the 7α-methoxy group weakens noncovalent binding and slows down both acylation and deacylation rates, but with major effect in the acylation rate, while elimination of the 3′ leaving group affects deacylation only. The criterion of trypsin sensitivity suggests that the 7α-methoxy group has a substantial effect on β-lactamase conformation at the acyl-enzyme stage but not on that of the initial noncovalent complex. The same criterion also indicates that the protein conformation of the inert acyl-enzymes generated on 3′ leaving group elimination is not significantly different from that of the native enzyme; the cause of their stability to hydrolysis is thus more subtle.

We have recently shown (Faraci & Pratt, 1984) that elimination of the 3′ leaving group accompanying β-lactamase-catalyzed hydrolyses of cephalosporin (**1**) antibiotics need not be concerted with β-lactam ring opening and thus that intermediate cephalosporoates (**2**) may be released from the enzyme into solution, where they spontaneously undergo an elimination reaction yielding **3** as the final product (Scheme I). Furthermore, we have shown (Faraci & Pratt, 1985) that in at least one case, that of the *Staphylococcus aureus* PC1 β-lactamase and cephalosporins with good 3′ leaving groups, X, the elimination reaction can occur at the enzyme active site, leading to a partitioning of the initial acyl-enzyme, **4**, between hydrolysis, completing turnover, and the elimination reaction, yielding a second acyl-enzyme, **5**, which then can hydrolyze, also completing turnover (Scheme II). Of particular interest was the observation that **5** hydrolyzed significantly more slowly than **4**, leading to transient inhibition of the enzyme. It seemed possible that this might be a general mechanism of β-lactamase inhibition, leading perhaps to more effective antibiotics. This mechanism probably explains, for example, the effectiveness of cephalothin and cephaloridine against β-lactamase-producing *S. aureus* (Wick, 1972). It may also be found relevant to mechanisms of stabilization of complexes of cephalosporins and cell wall D-alanine transpeptidases, thereby also leading to better antibiotics. Boyd (1984) has presented biological data suggesting that the presence of a 3′ leaving group does produce a more effective antibiotic.

Favorable partitioning of **4** to the potentially inert **5** would be influenced by the quality of the leaving group X, a point already demonstrated (Faraci & Pratt, 1985) and, presumably, also by the nature of the 7-substituent(s) and the β-lactamase. Combinations of the latter two factors giving rise to longer-lived acyl-enzymes would presumably promote the formation of **5** for a given leaving group.

Scheme I

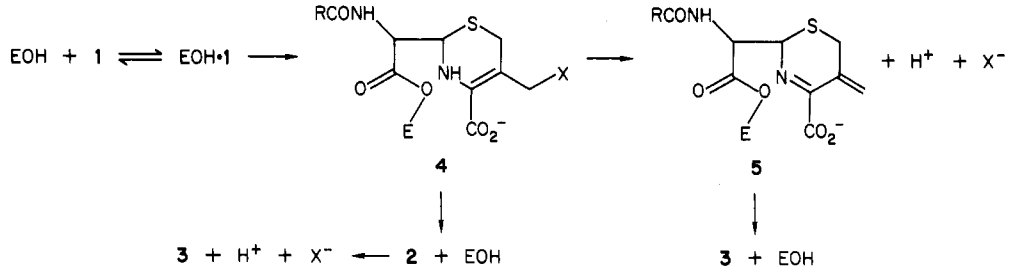


In this paper we examine the effect of 7α-methoxy substitution on the formation of **5**. This substituent has long been known to stabilize cephalosporins against β-lactamase-catalyzed hydrolysis (Stapley et al., 1972; Daoust et al., 1973; Gordon & Sykes, 1982) and has been included in several

† This work was supported by the National Institutes of Health.

0006-2960/86/0425-2934$01.50/0

Scheme II



clinically important cephamycin (7α-methoxycephalosporin, **6**) antibiotics, for example, cefoxitin, cefmetazole, and moxalactam. More recently, Fisher et al. (1980) have shown that the interaction of cefoxitin with RTEM-2 β-lactamase leads to a moderately stable acyl-enzyme. The cefoxitin molecule (**6d**) contains, however, not only the 7α-methoxy substituent but also a good (carbamoyloxy) 3′ leaving group. The question addressed here is of the relative contributions of these two substituents to the slow turnover of cefoxitin by RTEM-2 β-lactamase and the accumulation of the acyl-enzyme. The nature of the interaction of cefoxitin with *S. aureus* PC1 β-lactamase, another class A β-lactamase (Ambler, 1980), is also briefly assessed.

Experimental Procedures

Materials. PC1 β-lactamase from *S. aureus* and RTEM-2 β-lactamase from *Escherichia coli* W3310 were obtained from the Centre for Applied Microbiology and Research, Porton Down, England, and used as supplied. The specific activities of these enzymes against benzylpenicillin were respectively 110 units/mg at pH 7.50 and at 20 °C and 2750 units/mg at pH 7.00 and at 30 °C. Cephalothin, deacetylcephalothin, and deacetoxycephalothin were generous gifts of Eli Lilly and Co., and sodium cefoxitin was a gift of Merck Sharp & Dohme. Bovine pancreatic trypsin (type XI), orange peel acetylesterase, bovine liver L-glutamate dehydrogenase (type II), monosodium α-ketoglutarate, reduced nicotinamide adenine dinucleotide (NADH), and gelatin were purchased from Sigma Chemical Co. and used as supplied. All buffer materials were reagent grade.

3′-De(carbamoyloxy)cefoxitin (**6c**) was prepared from cefoxitin by the following method. Cefoxitin was first converted into the corresponding 3-*exo*-methylenecephem by the procedure of Ochiai et al. (1975). An NMR spectrum indicated that the product comprised about 95% methylenecephem and 5% cefoxitin. This material (200 mg) was dissolved in 1.0 mL of water to which pyridine (5.0 mL) was then added, and the ensuing solution was stirred for 17 days at 25 °C. The extended time period was required to ensure total destruction of the remaining cefoxitin. Purification of the desired product was achieved by means of a Sephadex QAE anion-exchange column (1.0 × 22 cm), eluted with a 0–0.5 M sodium chloride gradient. Acidification of the fractions containing cephalosporin (detected by 260-nm absorbance), extraction with ethyl acetate, and evaporation of the latter solvent led to pure **6c**, a colorless glass in 5% overall yield; ^{1}H NMR (^2H_2O, HCO_3^-) δ 1.90 (s, 3, CH_3), 3.10, 3.54 (AB, q, J = 17 Hz, 2, 2-H), 3.53 (s, 3, OCH_3), 3.98 (s, 2, Th CH_2), 5.13 (s, 1, H-7), 7.10 (m, 2, Th 3′,5′), 7.42 (m, 1, Th 4′).

3′-Decarbamoylcefoxitin (**6b**) was prepared by the following procedure. Cephalothin (the free acid, obtained as a precipitate by acidification of an aqueous solution of the sodium salt, and dried in vacuo) was suspended in ethyl acetate, 2 equiv of diphenyldiazomethane added, and the mixture stirred at room temperature for 4 h. The ethyl acetate mixture was then washed with 1 N HCl, water, saturated aqueous sodium bicarbonate, and saturated aqueous sodium chloride, dried, and evaporated to give the pure benzhydryl ester of cephalothin in quantitative yield. 7α-Methoxylation of the benzhydryl cephalothin was achieved by following the procedure of Koppel and Koehler (1973). Deprotection of the carboxyl group of the **6a** benzhydryl ester was carried out by dissolving the ester (0.6 g) into 25 mL of chloroform and adding 3.0 mL of trifluoroacetic acid. This solution was stirred for 15 min at room temperature after which it was evaporated to dryness. The residue was taken up in chloroform, extracted into aqueous sodium bicarbonate, and precipitated by acidification of the solution to pH 2 with HCl. The free acid was extracted into ethyl acetate. Evaporation of the dried extract yielded 0.3 g of product, which the NMR spectrum showed to be a 3:1 mixture of the required **6a** and cephalothin. **6a** was purified by our taking advantage of the fact that 7α-methoxycephalosporins are poor substrates of certain β-lactamases. Thus, the impure product mixture was dissolved in 20 mL of a 0.1 M potassium phosphate buffer solution (pH 7.5), 0.8 mL of a 13 μM RTEM-2 β-lactamase solution was added, and the solution was stirred for 30 min. The β-lactamase-catalyzed hydrolysis of cephalothin was followed to completion spectrophotometrically at 260 nm. The solution was then acidified and the remaining 7α-methoxycephalosporin isolated by extraction into ethyl acetate, which was subsequently removed by evaporation. It was then redissolved into water containing 1–2 equiv of sodium bicarbonate and purified by Sephadex QAE anion-exchange chromatography as described above, yielding the final product, **6a**, a colorless glass: ^{1}H NMR (^2H_2O, HCO_3^-) δ 2.10 (s, 3, –OAc), 3.37, 3.64 (AB, q, J = 18 Hz, 2, 2-H), 3.53 (s, 3, OCH_3), 3.98 (s, 2 Th CH_2), 4.78, 4.86 (AB q, J = 12 Hz, 2, $-CH_2OAc$), 5.15 (s, 1, H-7), 7.10 (m, 2, Th 3′,5′), 7.40 (m, 1, Th 4′).

Conversion of **6a** to the deacetyl derivative, **6b**, was achieved by hydrolysis of the acetate ester, catalyzed by orange peel acetylesterase. **6a** (50 mg) was dissolved in 4.0 mL of a 0.1 M ammonium bicarbonate buffer solution (pH 7.5) to which 200 μL of a 6.8 mg/mL acetylesterase suspension was added, and the mixture was stirred at 30 °C for 24 h. Purification of the desired product was achieved by application of the mixture to a Sephadex G25-40 column (1.0 × 22 cm), which was developed with water. Fractions (1.0 mL) were collected, and those containing cephalosporin (260-nm absorbance) were pooled and freeze-dried, yielding the sodium salt as a colorless powder: ^{1}H NMR (^2H_2O) δ 3.32, 3.62 (AB, q, J = 18 Hz, 2, 2-H), 3.53 (s, 3, OCH_3), 3.98 (s, 2, Th CH_2), 4.26 (s, 2, $-CH_2OH$), 5.17 (s, 1, H-7), 7.10 (m, 2, Th, 3′,5′), 7.41 (m, 1, Th 4′).

Analytical Methods. All kinetics experiments involving RTEM-2 β-lactamase were done at 30 °C in 0.1 M potassium phosphate buffer at pH 7.0 and those involving *S. aureus* PC1 β-lactamase at 20 °C in 0.1 M potassium phosphate buffer

at pH 7.50, 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). The β-lactamase concentrations were determined spectrophotometrically, assuming that 1.99×10^4 M^{-1} cm^{-1} was the extinction coefficient of the *S. aureus* enzyme at 276.5 nm (Carrey & Pain 1978) and 2.94×10^4 M^{-1} cm^{-1} was the extinction coefficient of the RTEM-2 enzyme at 280 nm (Fisher et al., 1980).

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 under steady-state conditions. The reactions were followed spectrophotometrically in a 2-mm path length cuvette at 289 nm for cefoxitin and **6a**, at 288 nm for **6c**, and at 292.5 nm for **6b**. The concentration of RTEM-2 β-lactamase was between 6.0 and 9.0 μM while substrate concentrations were between 2 and 11 mM for **6b** and **6c** and between 0.40 and 6.0 mM for cefoxitin and **6a**. Michaelis–Menten parameters were also obtained by this method for cefoxitin with *S. aureus* PC1 β-lactamase. The concentration of substrate was varied between 9 and 450 μM while the concentration of enzyme was 8.5 μM.

Detection of Acyl-Enzyme Intermediates. Long-lived acyl-enzyme intermediates were detected by dilution of an aliquot from an incubation mixture of enzyme, close to saturated with cephalosporin, into an assay cuvette containing a large excess of the excellent substrate benzylpenicillin. The initial velocity of the benzylpenicillin hydrolysis was taken to be a direct measure of the amount of free and rapidly reversibly bound enzyme present in the incubation mixture. Thus, to a 100-μL sample of cephalosporin (concentrations used were 16.0, 15.0, 30.0, and 32.0 mM for cefoxitin, **6a**, **6c**, and **6b**, respectively) was added 5 μL of the RTEM-2 enzyme (final enzyme concentration, 1.0 μM). An incubation of the mixture for 20 min at 30 °C to ensure attainment of the steady state was followed by addition of a 5-μL aliquot of this mixture to 3.0 mL of a 2.0 mM benzylpenicillin assay cuvette. Calculations showed that only about 13% of the cefoxitin was consumed during the incubation. The hydrolysis of benzylpenicillin was followed at 240 nm. Similarly, when *S. aureus* PC1 β-lactamase (12 μM) was incubated with cefoxitin (0.22 mM) at 20 °C for 30 min (during which time about 16% of the cefoxitin was consumed), a 20-μL aliquot was assayed as above.

Effect of Trypsin on Incubation Mixtures. The effect of trypsin on the β-lactamases upon incubation with the various cephamycins was determined in a fashion similar to that of Citri and Zyk (1982) and Citri et al. (1984). To 50 μL of a cephamycin solution (final concentrations were 15.5, 18.0, and 32.0 mM for cefoxitin, **6c**, and **6b**, respectively) was added 10 μL of an RTEM-2 β-lactamase solution (final concentration, 3.07 μM), followed by 5 μL of a trypsin solution (final concentration, 0.4 mg/mL). An incubation of the mixture for 15 min at 30 °C was followed by addition of a 10-μL aliquot into an assay cuvette containing 3 mL of a 2.0 mM benzylpenicillin solution. The hydrolysis of benzylpenicillin was followed at 240 nm. Controls included identically treated samples of the enzyme alone, of enzyme and trypsin without cephalosporin, and of enzyme and cephalosporin without trypsin. The effect of trypsin on *S. aureus* PC1 β-lactamase was ascertained by a similar method. To a 50-μL solution of the *S. aureus* enzyme (at a concentration of 23 μM) was added 5 μL of a cephalosporin solution (final concentrations of 0.215 mM for cefoxitin and 24.6 μM for cephalothin were used) and 10 μL of a trypsin solution (to a final concentration of 0.92 mg/mL). The mixture was incubated at 20 °C for 30 min to reach the steady state, and then a 5-μL aliquot was diluted into 0.5 mL of 0.1 M phosphate buffer at pH 7.5 containing 0.2% gelatin. The diluted mixture was incubated for either 80 min (cephalothin) or 9 h (cefoxitin) to achieve essentially complete deacylation of the enzyme complex. The restored activity was then measured by assay of a 200-μL aliquot against benzylpenicillin. Controls were also set up as above.

Isolation of an Acyl-Enzyme. A 6.0-mg portion of sodium cefoxitin was added to 0.85 mL of a 0.8 M phosphate buffered (pH 7.0) solution at 30 °C containing 90 μM of the RTEM-2 β-lactamase. After about 6 min, the mixture was loaded onto a Sephadex G25-40 column (1.0 × 22 cm) at 4 °C and eluted with a flow rate of 0.667 mL/min with 20 mM potassium phosphate buffer at pH 7.0 and at the same low temperature. Fractions of 0.5 mL were collected and those containing enzyme identified through their absorbance at 280 nm.

Assay for the Hydrolysis Product of Cefoxitin. A previously described method (Faraci & Pratt, 1985) for the determination of hydrolyzed cephalosporins that contain good leaving groups at the 3′-position was used. It was found that incubation of cefoxitin, or its hydrolysis product, in 0.8 M sodium hydroxide for 30 min at 30 °C led to an absorption peak at 320 nm ($\epsilon = 1900$ M^{-1} cm^{-1}) whose intensity was linear with the cefoxitin concentration up to at least 100 μM.

Assay for Ammonia. The method of Buttery and Rowsell (1971) was used, in which the enzyme glutamate dehydrogenase utilizes ammonium ion in the NADH-dependent reduction of α-ketoglutarate to give L-glutamate. A 0.25-mL aliquot from the column fractions described above was placed in a 0.5-mL, 1-cm path length cuvette containing an ammonia assay mixture, preincubated at 37 °C, consisting of 0.10 mL of 20 mM potassium phosphate buffer at pH 7.0, 0.135 mL of glycerol, 5 μL of a 14 mM NADH solution, 5 μL of a 0.83 M α-ketoglutarate solution, and 5 μL of a 133 mg/mL glutamate dehydrogenase solution. The decrease in absorbance at 340 nm corresponding to the oxidation of NADH was followed for 30 min. A calibration curve using ammonium chloride exhibited linearity of this absorption decrease up to a concentration of 30 μM and was used to determine the amount of ammonia in each fraction.

^1H NMR Spectra of Reaction Products. Small portions of solid RTEM-2 β-lactamase were added to NMR tubes containing sodium cefoxitin (10 mM) and sodium bicarbonate (20 mM) in ^2H_2O. NMR spectra were then recorded at appropriate intervals.

Results

Nature of the Acyl-Enzyme. ^1H NMR spectra showed that RTEM-2 β-lactamase catalyzed hydrolysis of cefoxitin yielded the β-lactam ring-opened 7α-methoxy-*exo*-methylenethiazine **3** (R′ = OMe). The NMR spectrum of **3** (R′ = OMe) observed here [(^2H_2O) δ 3.22 (s, 3, OCH_3), 3.50, 3.65 (AB q, J = 14 Hz, 2, 2-H), 3.90, 4.02 (AB q, J = 16 Hz, 2, Th CH_2), 5.16 (s, 1, H-7), 5.64 (s, 1, =CH), 5.68 (s, 1, =CH), 7.10 (m, 2, Th 3′,5′), 7.41 (m, 1, Th 4′)] is in accord with spectra of hydrolysis products of cephalosporins with good 3′ leaving groups (Faraci & Pratt, 1985). Thus β-lactam ring cleavage in cephamycins is also accompanied by elimination of the 3′ leaving groups.

Knowles and co-workers (Fisher et al., 1980) have demonstrated that the hydrolysis of cefoxitin catalyzed by RTEM-2 β-lactamase proceeds via an acyl-enzyme intermediate [Scheme III, where ES′ is the acyl-enzyme; the numbers

Table I: Analysis of Column Fractions during Isolation of the Covalent RTEM-2/Cefoxitin Adduct

fraction	concn (μM)		
	enzyme	cefoxitin	ammonia[a]
1	0	0	0
2	20.4	12	2.0[b]
3	21.4	16	2.0[b]
4	9.7	6.5	1.0[b]
5	0	0	0

[a] Above a buffer background of ca. 2 μM. [b] Control experiments showed that this was free ammonia occluded on the enzyme from the incubation mixture and carried down the column.

are taken from Fisher et al. (1980)]. Since we have now established that the 3′ leaving group is not present in P, the question arose as to whether it is present in ES′; i.e., does the elimination of the carbamoyloxy group occur at the enzyme active site or subsequently in solution? In order to decide on this question, which Fisher et al. (1980) did not address, ES′ was isolated by exclusion chromatography, as described under Experimental Procedures. The results of various analyses of relevant column fractions are given in Table I.

Scheme III

$$E + S \underset{K_s = 3.9\ mM}{\rightleftharpoons} ES \xrightarrow{k_2 = 0.024\ s^{-1}} ES' \xrightarrow{k_3 = 0.0048\ s^{-1}} E + P$$

The isolated acyl-enzyme was about 30% active as soon as it could be assayed after coming off the column. This is not unreasonably high—a calculation based on the rate constants, given in Scheme III, shows that the amount of E + ES should be 18% of the total enzyme at saturating cefoxitin concentrations.

Column fractions were also immediately assayed for cefoxitin [or its hydrolysis product(s)] by the hydroxide method described above. These assays indicated, as seen in Table I, that cefoxitin residues were associated with the enzyme, with cefoxitin concentrations between 60% and 70% that of protein concentrations. This result, along with the activity data, suggests that the inactive (toward benzylpenicillin) acyl-enzyme has 1 equiv of the cefoxitin nucleus covalently bound to it. Fisher et al. (1980) of course reached the same conclusion.

A difference absorption spectrum (Figure 1) was generated by subtracting from the spectrum of one of the acyl-enzyme fractions a spectrum of the native enzyme at the same concentration. The extinction coefficient scale on this plot was calculated by assuming the concentration of the absorbing species to be 65% of the enzyme concentration. Also shown in Figure 1 is the spectrum of **3** (R′ = OMe). It is clear that the enzyme-bound chromophore in ES′ resembles the chromophore of **3** (R′ = OMe), which strongly suggests that elimination of the 3′-carbamoyloxy group had occurred in forming ES′. If the leaving group had not been eliminated, the spectrum should have been similar to that of hydrolyzed **6c** with an absorption maximum at 262 nm (ϵ = 4000 M^{-1} cm^{-1}).

This conclusion was confirmed by assay of the column fractions for ammonia after the acyl-enzyme had completely hydrolyzed. If the leaving group were still present in ES′, ammonia would be released after deacylation since the observed cephalosporin product is **3** (R′ = OMe). Any eliminated carbamate should decarboxylate to yield ammonia under the conditions of the experiment (Johnson & Morrison, 1972); indeed, the ammonia assay showed that 1 equiv of ammonia was released per cefoxitin molecule hydrolyzed. The absence

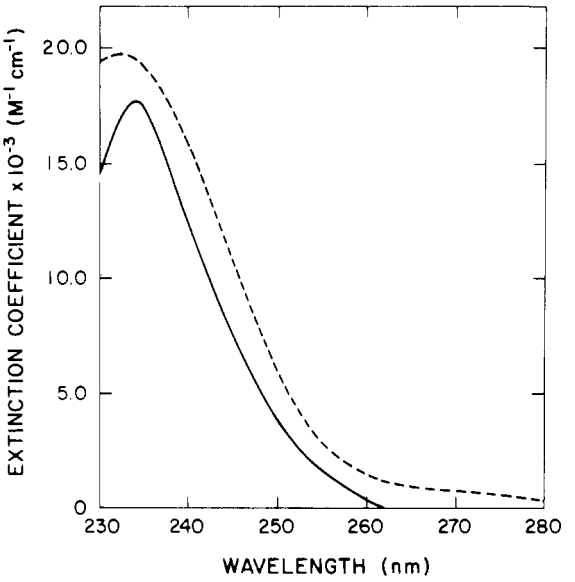


FIGURE 1: Difference absorption spectrum (vs. free enzyme) of the covalent complex of cefoxitin and RTEM-2 β-lactamase, prepared as described in the text (—). Also shown is the spectrum of **3** (---).

of ammonia above background in the column fractions (Table I) shows clearly that the carbamoyloxy group has already been lost in ES′.

Comparisons with Cephamycins Lacking a 3′ Leaving Group. In order to separate the effects of the 3′ leaving group from those of the 7α-methoxy group, compounds **6b** and **6c** were prepared, which have a 3′-OH and a 3-H, respectively. It was anticipated that the hydroxyl constituent of **6b** would not be eliminated at the enzyme active site, and the results reported below tend to support this. [Although early observations by Abraham and co-workers (Hamilton-Miller et al., 1970a,b) suggest that P99 β-lactamase might catalyze elimination of the 3′-hydroxyl group, our recent model studies (Pratt & Faraci, 1986) indicate that these experiments were misinterpreted and that class A and C β-lactamases do not catalyze this reaction.]

When RTEM-2 β-lactamase was incubated with saturating concentrations of cefoxitin or **6a** (i.e., cephamycins that contain good 3′ leaving groups) until steady state was achieved and then diluted into an assay mixture containing benzylpenicillin, an initial rate of benzylpenicillin hydrolysis that was roughly 20% of full activity was observed. This is as expected from the data of Fisher et al. (1980), as explained above. The rate then increases in a first-order manner to full activity. The initial activity can be interpreted (Fisher et al., 1980; Scheme III) as the amount of E + ES, while the return of activity curve corresponds to the breakdown of ES′ to regenerate E. Since the value of the first-order rate constant for return of activity is essentially the same as that of k_{cat}, it is clear, as implied by Scheme I, that breakdown of ES′ (deacylation probably) is rate-determining to steady-state turnover. We observed the same results with **6a**.

In strong contrast, when this experiment was carried out with compounds **6b** or **6c**, cephamycins that lack a good 3′ leaving group, no change from the initial activity was observed on dilution into the benzylpenicillin assay. In these cases no long-lived acyl-enzyme can have accumulated.

Steady-state parameters for the hydrolysis of the cephamycins **6a–d** by RTEM-2 β-lactamase are given in Table II. Data for the cephalosporins **1a–c** are included for comparison.

Effect of Trypsin on RTEM-2 β-Lactamase in the Presence of Cephamycins. The results of this study are given in Table

Table II: Steady-State Parameters and Derived[a] Constants for Hydrolysis of Cephamycins and Analogous Cephalosporins Catalyzed by RTEM-2 β-Lactamase[b]

substrate	k_{cat} (s^{-1})	K_m (mM)	K_s (mM)	k_2 (s^{-1})	k_3 s^{-1})
cefoxitin (**6d**)	0.0041	0.90	4.0[c]	0.024[c]	0.0048[c]
7α-methoxycephalothin (**6a**)	0.0051	0.85	*d*	*d*	*d*
3′-decarbamoylcefoxitin (**6b**)	0.0141	10.8	10.8	0.0141	*e*
3′-de(carbamoyloxy)cefoxitin (**6c**)	0.0020	6.0	6.0	0.0020	*e*
cephalothin (**1a**)	131	0.27	0.27	131	*e*
3′-deacetylcephalothin (**1b**)	32	0.65	0.65	32	*e*
3′-deacetoxycephalothin (**1c**)	17	0.55	0.55	17	*e*

[a] Derived as described in the text. [b] Reaction conditions in text. [c] Taken from Fisher et al. (1980). [d] Not determined. [e] $>k_2$.

Table III: Effect of Trypsin on the Activity of RTEM-2 β-Lactamase in the Presence of Cephamycins[a]

incubation mixture	rel β-lactamase act.
enzyme	1.0
enzyme/trypsin	0.85
enzyme/cefoxitin	0.62
enzyme/cefoxitin/trypsin	0.08
enzyme/**6b**	0.96
enzyme/**6b**/trypsin	0.65
enzyme/**6c**	0.96
enzyme/**6c**/trypsin	0.78

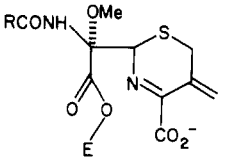
[a] See text for experimental details.

III. It is clear that the presence of a good 3′ leaving group in the cephamycin promotes proteolytic digestion of the enzyme by trypsin.

Effect of Cefoxitin on PC1 β-Lactamase. Steady-state kinetic parameters for cefoxitin hydrolysis catalyzed by PC1 β-lactamase were K_m = 19.0 μM and k_{cat} = 3.0 × 10^{-4} s^{-1}. Incubation of the β-lactamase with saturating concentrations of cefoxitin followed by dilution of this mixture into a benzylpenicillin assay cuvette gave rise to an initial activity toward benzylpenicillin of essentially zero. The activity returned in a first-order manner with a rate constant, derived as previously described (Faraci & Pratt, 1985), of 1.6 × 10^{-4} s^{-1}. Thus deacylation of an inert acyl-enzyme intermediate is probably rate-determining to steady-state turnover at saturation. The effect of trypsin on the activity of PC1 β-lactamase in the presence of cefoxitin and cephalothin is given in Table IV.

Discussion

The results show that the long-lived acyl-enzyme intermediate formed on interaction of cefoxitin and RTEM-2 β-lactamase, and first described by Knowles and co-workers (Fisher et al., 1980), has the structure **7**, where the feature discovered



7

and emphasized in this work is that elimination of the 3′-carbamoyloxy leaving group has occurred at the enzyme active site. The sequence of events in turnover of this substrate is thus as given in Scheme II. Since the rate constant for breakdown of the acyl-enzyme **7** is essentially the same as for steady-state turnover under saturating conditions, the pathway must be very close to linear with little or no competing hydrolysis of 7α-methoxy-**4** prior to elimination. This is of course the same sequence that we have shown to hold for the turnover of cephalosporins with good 3′ leaving groups, e.g., cephalothin, by *S. aureus* PC1 β-lactamase (Faraci & Pratt, 1985). The accumulating acyl-enzyme, **5**, in the latter case is also slow to hydrolyze.

Table IV: Effect of Trypsin on the Activity of PC1 β-Lactamase in the Presence of Cephalothin and Cefoxitin

incubation mixture	rel β-lactamase act.
enzyme	1.0
enzyme/trypsin[a]	0.66
enzyme/cephalothin[a]	0.95
enzyme/cephalothin/trypsin[a]	0.78
enzyme/trypsin[b]	0.44
enzyme/cefoxitin[b]	0.39
enzyme/cefoxitin/trypsin[b]	0.009

[a] 80-min incubation. See text for experimental details. [b] 9-h incubation. See text for experimental details.

Thus, two structural elements are present in the cefoxitin molecule that can, potentially at least, lead to impaired turnover by β-lactamases, the 7α-methoxy group and a good 3′ leaving group. The former of these has long been known to have this effect (Stapley et al., 1979; Daoust et al., 1973; Gordon & Sykes, 1982), but the potential of the latter has only recently been recognized (Faraci & Pratt, 1985). Sufficient is now known about the turnover process to permit dissection apart of the contributions of the two functionalities; the remaining experiments described here were designed to do this.

Let us take first the 3′ leaving group and RTEM-2 β-lactamase. Now that it is known that the 3′-carbamoyloxy group is eliminated at the RTEM-2 active site, the contribution of this reaction to the slow deacylation can be assessed through comparison of the kinetics of cefoxitin (or 7α-methoxycephalothin, **6a**, which appears to behave in an essentially identical manner) hydrolysis with those of 3′-decarbamoyl (**6b**) and 3′-de(carbamoyloxy)cefoxitin (**6c**). Although the steady-state parameters (Table II) do not in themselves suggest significant differences, except perhaps somewhat weaker binding ($1/K_m$), in the absence of a good 3′ leaving group, they do gain greater stature on consideration of the results of the experiments designed to detect long-lived acyl-enzymes. These experiments showed us, as they did Fisher et al. (1980), that such an intermediate accumulated during turnover of cefoxitin and that deacylation of this intermediate was rate-determining at saturation. In strong contrast, however, no such intermediate was detected in turnover of **6b** and **6c**. In these cases, therefore, taking into account the values of k_{cat}, deacylation could not be rate-determining at saturation and therefore acylation presumably was; at saturation the dominant enzyme–substrate complex must be the rapidly dissociating Michaelis–Menten complex; $k_{cat} = k_2$ and $K_m = K_s$. Thus the K_s values of cefoxitin, **6b**, and **6c** and the acylation rate constants, k_2, are given in Table II (the cefoxitin figures are from Scheme III). The order of k_2 values is as might be anticipated on the basis of the decrease in electron-withdrawing power of the 3′-substituents in the series and agrees well with the order of the second-order rate constants for hydroxide ion catalyzed hydrolysis [0.075, 0.047, and 0.010 s^{-1} M^{-1}, respectively; obtained from spectrophotometrically determined hydrolysis rates in potassium hydroxide solutions (μ = 0.1) at 30 °C]. Thus the presence of a small 3′ leaving group does not significantly

affect either binding or acylation rates, except of course for its electronic effects on the latter (Boyd, 1982).

The deacylation rate constants clearly and unexpectedly (on the basis of electronic effects) reflect the presence of a 3′ leaving group, however. Since no transient intermediate was detected for either **6b** or **6c**, the k_3 values in these cases must be at least $10k_2$, i.e., $10k_{cat}$, or 0.14 s^{-1} and 0.02 s^{-1}, respectively. Thus, taking the former upper estimate, and comparing it with k_3 for cefoxitin, we see that the 3′ leaving group in cefoxitin, presumably because of its elimination at the enzyme active site as in Scheme II, reduced the deacylation rate constant at least 30-fold.

The contribution of the 7α-methoxy group can now be addressed. It is clear from the data of Table II that cefoxitin (and **6a**) will hydrolyze much more slowly than cephalothin at all substrate concentrations. The same is true of the other cephamycins in Table II with respect to their 7α-unsubstituted analogues. Assuming that enzyme acylation is rate-determining at saturation by cephalothin and the other 7α-unsubstituted analogues, i.e., $k_{cat} = k_2$ (this appears to be likely in view of the comparison of their K_m and k_{cat} values, particularly the latter, with the K_s and k_2 values of their respective 7α-methoxy analogues, and with the alkaline hydrolysis rates discussed above), then introduction of the 7α-methoxy group can be seen to retard acylation by a factor of around 5×10^3 and thus deacylation apparently by something greater than 5×10^4. The latter effect is only "apparently" due to the 7α-methoxy group since the contribution of the 3′ leaving group on deacylation has not been taken into account in this number. It might be noted in passing that the addition of a 7α-methoxy group to a cephalosporin does not significantly affect its reactivity with nucleophiles (Indelicato & Wilham, 1974; Narisada et al., 1984); the change in acylation rates must therefore reflect changes in the structure of the enzyme–substrate complex, as discussed below.

The question remaining then is just how much of the greater than 5×10^4 retardation of deacylation of the RTEM-2 enzyme is due to the 7α-methoxy group and how much to 3′-elimination. The discussion above suggests that a factor of at least 30 is due to the latter, but that leaves a factor of greater than 10^3 still unassigned. The only direct evidence on this point comes from consideration of the data available for the *S. aureus* PC1 enzyme. Hydrolysis of 3′-deacetoxycephalothin in the presence of this enzyme occurs with $k_{cat} = 1.0$ s^{-1} (Faraci & Pratt, 1985). Since no transient intermediate with appropriate half-life was observed on dilution, this rate probably represents enzyme acylation; i.e., $k_2 = 1.0$ s^{-1} and thus $k_3 > 1$ s^{-1}. Since the deacylation rate of the cefoxitin complex is 1.5×10^{-4} s^{-1}, the 7α-methoxy group plus 3′-elimination retards deacylation by $>10^4$, a result comparable to that observed with the RTEM-2 enzyme. However, since the rate of deacylation of the cephalothin complex after 3′-elimination is 10^{-3} s^{-1} (Faraci & Pratt, 1985), the presence of the 7α-methoxy group contributes only a factor of 10 to this number. This comparison suggests that in the case of the PC1 enzyme the dominant factor in the slow deacylation of the cefoxitin complex is the 3′-elimination reaction rather than the presence of the 7α-methoxy group. This may be true for RTEM-2 β-lactamase also. It should be borne in mind of course, particularly in view of the evidence below, which indicates that the protein conformation in 7α-methoxylated acyl-enzymes might be significantly different from that of 7α-unsubstituted analogues, that the effects of the 7α-methoxy group and the elimination reaction may not be directly additive, i.e., that the elimination reaction might have a quantitatively different effect on the lability of these two types of complex.

It remains now to consider the mechanisms by which the 7α-methoxy and 3′ leaving group bring about their effects. Previous thoughts on the effect of the 7α-methoxy group on β-lactamase stability have usually involved ideas of steric hindrance at the active site (Gordon & Sykes, 1982), but until now the actual step or steps at which the effect was expressed could not be determined. In the present case of RTEM-2 β-lactamase the results discussed above indicate first that the 7α-methoxy group does weaken binding ($1/K_s$, Table II) to the enzyme, by a factor of between 10 and 20. This may well be a steric effect. It is not known however from the K_s values alone whether the orientation of the cephem nucleus and substituents are exactly the same in the complexes with and without 7α-methoxy substitution, nor whether the protein conformation is exactly the same. With respect to the latter point however the findings of Citri and Zyk (1982) and Citri et al. (1984) should be mentioned. They have shown that, in the presence of cefoxitin, the RTEM-1 enzyme [which differs from RTEM-2 in one amino acid residue, but not significantly in any catalytic property yet observed (Ambler, 1980)] becomes more susceptible to proteolytic digestion by pronase and trypsin, which suggests that this substrate induces a different and perhaps more open or mobile conformational structure. This result of course resembles in many ways the earlier results of Citri and co-workers [see Citri (1981), for example] with class A (Ambler, 1980) β-lactamases and the A type penicillins, which, like cefoxitin perhaps, have bulky and rigid substituents (Samuni & Meyer, 1978; Blanpain et al., 1980) adjacent to the β-lactam carbonyl group. With the A type penicillins at least, it seems likely that the more open and fragile structure is present in the acyl-enzyme (Kiener & Waley, 1977; Kiener et al., 1980) although its presence at other stages may not be excluded.

In the present work with cefoxitin and RTEM-2 β-lactamase we first confirmed the result of Citri and Zyk (1982) and Citri et al. (1984) that cefoxitin does induce trypsin sensitivity (Table III). Since the major enzyme species present at the cefoxitin concentrations used would be the acyl-enzyme **7**, and in view of the work on the A type penicillins referred to above, it seems likely that the trypsin-sensitive form might well be **7**. In support of this proposition it was found (Table III) that the cephamycins **6b** and **6c** which lack a good 3′ leaving group and consequently an accumulating acyl-enzyme have much less effect on the trypsin sensitivity of RTEM-2 β-lactamase. In these cases the predominant form of the enzyme in solution would have been the Michaelis complex. It would seem then that the initial binding of the cephamycins may have much less effect on the protein conformation than does the acylation reaction. The small effect observed with **6b** and **6c** may in fact also occur at the acyl-enzyme stage. The implication of this result seems to be that during the acylation of the enzyme by cephamycins the 7α-methoxy group induces a significant conformational change. This may occur concerted with, or rapidly subsequent to, acylation, and may or may not be an extension of a conformational change that may normally accompany acylation. Since the rate of enzyme acylation by cefoxitin is much slower than that by cephalothin, it is clear that the 7α-methoxy group must also affect the covalent chemistry. This may reflect a somewhat different orientation of binding by the cephamycins, the negative (steric) effect of the methoxy group on an essential conformation change during acylation, or an abnormal and difficult conformational change required in order to achieve acylation by cephamycins. Un-

doubtedly the two phenomena, the abnormal conformational change and slow acylation, are correlated, however.

The results of the trypsin proteolysis of the PC1 β-lactamase are also instructive. Here, both with cephalothin and cefoxitin, deacylation is much slower than acylation, and the proteolysis is almost certainly of the accumulated acyl-enzyme. The relevant data (Table IV) suggest that, as with RTEM-2 β-lactamase, cefoxitin induces a conformational change on acylation of the enzyme leading to enhanced trypsin susceptibility. Since PC1 β-lactamase is also a class A β-lactamase with significant sequence homology to the RTEM-2 enzyme (Ambler, 1980), the same conformational change may be involved. On the other hand, the trypsin susceptibility of the cephalothin adduct of PC1 is not different from that of the native enzyme. This suggests that the structural change in these β-lactamases on elimination of the 3′ leaving group from the acyl-enzyme, leading to the slow hydrolysis of **5** and **7**, is different from and probably more subtle than the change brought about by the 7α-methoxy substituent of the cephamycins. It is not wholly impossible that the β-lactamase active site conformation present in these inert acyl-enzymes after the elimination reaction may be related to that present at the active sites of the cell wall D-alanine transpeptidase, which also, of course, form inert acyl-enzymes with β-lactams. Such a conformation would have been selected against in β-lactamase evolution but may still be accessible by selective substrate induction. The crystallographic studies under way on both of these classes of enzymes [see, for example, Kelly et al. (1985) and references cited therein] may eventually permit a decision on this point.

In view of these and our previous results (Faraci & Pratt, 1985) it seems that formation of a more hydrolytically inert acyl-enzyme through departure of the 3′ leaving group of a cephem may be a general property of the active sites of the class A β-lactamases. The 7α-methoxy substituent of cephamycins favors formation of this acyl-enzyme probably mainly because of its effect on deacylation rates (and despite the fact that its effect on acylation rates is probably greater)—a slowly hydrolyzing acyl-enzyme will allow time for the elimination to occur. Rearrangement to a similar inert structure may be generally possible for the acyl-enzymes derived from penems and carbapenems also (Charnas & Knowles, 1981; Easton & Knowles, 1982; Faraci & Pratt, 1985; Knowles, 1985). It may also contribute toward the stability of covalent complexes of certain cephalosporins and cephamycins (Bush et al., 1982; Labia et al., 1983; Murakami & Yoshida, 1985) with class C β-lactamases. As discussed previously (Faraci & Pratt, 1985), the inert nature of these species must derive from enzyme conformational changes at the active site; the difference spectra of the cephalosporin complexes (Figure 1, for example) indicate that no further covalent interaction with the enzyme occurs.

The incidence of inert intermediates would seem on one hand to reduce the effectiveness of the β-lactamase toward these β-lactams and thus increase their effectiveness as antibiotics. On the other hand, however, this mechanism does increase the affinity of the enzyme for the β-lactam (K_m is decreased), and although k_{cat} may be much smaller after the rearrangement, in some cases at least, k_{cat}/K_m may in fact be comparable or higher than in the cases where elimination does not occur. This would mean that the enzyme would be more effective at low antibiotic concentrations and thus perhaps more effective in vivo (Vu & Nikaido, 1985). (It is not easy and probably not possible to determine just what β-lactam a given β-lactamase is optimally evolved to defend against.) An enzyme that permitted or encouraged the rearrangement might also be more effective in the proposed antibiotic trapping or "nonhydrolytic barrier" mechanism of β-lactam resistance (Then & Angehrn, 1982; Gutman & Williamson, 1983; Sanders & Sanders, 1985; Yoshimura & Nikaido, 1985) since a strong affinity for β-lactams must be a prerequisite for this mechanism to be effective.

ACKNOWLEDGMENTS

We thank Drs. B. G. Christensen and J. A. Webber for their kind assistance in our obtaining several of the β-lactams used in this research.

REFERENCES

Ambler, R. P. (1980) *Philos. Trans. R. Soc. London, B 289*, 321–331.

Blanpain, P. C., Nagy, J. B., Laurent, G. H., & Durant, F. V. (1980) *J. Med. Chem. 23*, 1283–1292.

Boyd, D. B. (1982) in *Chemistry and Biology of β-Lactam Antibiotics* (Morin, R. B., & Gorman, M., Eds.) Vol. 1, pp 437–545, Academic Press, New York.

Bush, K., Freudenberger, J. S., & Sykes, R. B. (1982) *Antimicrob. Agents Chemother. 22*, 414–420.

Buttery, P. J., & Rowsell, E. V. (1971) *Anal. Biochem. 39*, 297–310.

Charnas, R. L., & Knowles, J. R. (1981) *Biochemistry 20*, 2732–2737.

Citri, N. (1981) in *β-Lactam Antibiotics* (Mitsuhashi, S., Ed.) pp 225–232, Springer-Verlag, New York.

Citri, N., & Zyk, N. (1982) *Biochem. J. 201*, 425–427.

Citri, N., Kalkstein, A., & Zyk, N. (1984) *Eur. J. Biochem. 144*, 333–338.

Daoust, D. R., Onishi, H. R., Wallick, H., Hendlin, D., & Stapley, E. O. (1973) *Antimicrob. Agents Chemother. 3*, 254–261.

Easton, C. J., & Knowles, J. R. (1982) *Biochemistry 21*, 2857–2862.

Faraci, W. S., & Pratt, R. F. (1984) *J. Am. Chem. Soc. 106*, 1489–1490.

Faraci, W. S., & Pratt, R. F. (1985) *Biochemistry 24*, 903–910.

Fisher, J., Belasco, J. G., Khosla, S., & Knowles, J. R. (1980) *Biochemistry 19*, 2895–2901.

Gordon, E. M., & Sykes, R. B. (1982) in *Chemistry and Biology of β-Lactam Antibiotics* (Morin, R. B., & Gorman, M., Eds.) Vol. 1, pp 199–370, Academic Press, New York.

Gutman, L., & Williamson, R. A. (1983) *J. Infect. Dis. 148*, 316–321.

Hamilton-Miller, J. M. T., Newton, G. G. F., & Abraham, E. P. (1970a) *Biochem. J. 116*, 371–384.

Hamilton-Miller, J. M. T., Richards, E., & Abraham, E. P. (1970b) *Biochem. J. 116*, 385–395.

Indelicato, J. M., & Wilham, W. L. (1974) *J. Med. Chem. 17*, 1577–1578.

Johnson, S. L., & Morrison, D. L. (1972) *J. Am. Chem. Soc. 94*, 1323–1334.

Kelly, J. A., Knox, J. R., Moews, P. C., Hite, G. J., Bartolone, J. B., Zhao, H., Joris, B., Frère, J.-M., & Ghuysen, J.-M. (1985) *J. Biol. Chem. 260*, 6449–6458.

Knowles, J. R. (1985) *Acc. Chem. Res. 18*, 97–104.

Koppel, G. A., & Koehler, R. E. (1973) *J. Am. Chem. Soc. 95*, 2403–2404; U.S. Patent 3 994 885.

Labia, R., Morand, A., & Peduzzi, J. (1983) *J. Antimicrob. Chemother. 11* (*Suppl. A*), 153–157.

Murakami, K., & Yoshida, T. (1985) *Antimicrob. Agents Chemother. 27*, 727–732.

Narisada, M., Yoshida, T., Ohtani, M., Ezumi, K., & Takasuka, M. (1984) *J. Med. Chem. 26*, 1577–1582.
Ochiai, M., Aki, O., Morimoto, A., Okada, T., & Morita, K. (1975) *Tetrahedron 31*, 115–122.
Pratt, R. F., & Faraci, W. S. (1986) *J. Am. Chem. Soc.* (in press).
Samuni, A., & Meyer, A. Y. (1978) *Mol. Pharmacol. 14*, 704–709.
Sanders, C. C., & Sanders, W. E., Jr. (1985) *J. Infect. Dis. 151*, 399–406.
Stapley, E. O., Jackson, M., Hernandez, S., Zimmerman, S. B., Currie, S. A., Mochales, S., Mata, J. M., Woodruff, H. B., & Hendlin, D. (1972) *Antimicrob. Agents Chemother. 2*, 122–131.
Then, R. L., & Angehrn, P. (1982) *Antimicrob. Agents Chemother. 21*, 711–717.
Vu, H., & Nikaido, H. (1985) *Antimicrob. Agents Chemother. 27*, 393–398.
Waley, S. G. (1974) *Biochem. J. 139*, 789–790.
Wick, W. E. (1972) in *Cephalosporins and Penicillins* (Flynn, E. H., Ed.) pp 496–531, Academic Press, New York.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324–332.
Yoshimura, F., & Nikaido, H. (1985) *Antimicrob. Agents Chemother. 27*, 84–92.

Inhibition of *Euglena gracilis* and Wheat Germ Zinc RNA Polymerases II by 1,10-Phenanthroline Acting as a Chelating Agent†

B. Mazus, K. H. Falchuk, and Bert L. Vallee*

Center for Biochemical and Biophysical Sciences and Medicine and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

Received December 12, 1985; Revised Manuscript Received January 22, 1986

ABSTRACT: Copper complexes of 1,10-phenanthroline (OP–Cu) hydrolyze DNA [D'Aurora, V., Stern, A. M., & Sigman, D. S. (1978) *Biochem. Biophys. Res. Commun. 80*, 1025–1032; Marshall Pope, L., Reich, K. A., Graham, D. R., & Sigman, D. S. (1982) *J. Biol. Chem. 257*, 12121–12128]. This reaction has been studied to determine whether the 1,10-phenanthroline (OP) inhibition of the activity of RNA and DNA polymerases is the result of template hydrolysis or the chelation of a metal associated with and essential to the function of these enzymes. Addition of 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) to DNA generates a fluorescence signal with a linear increase of the intensity over a broad range of DNA concentrations from 0 to 100 μg/mL. The progress of hydrolysis of DNA by DNase I or OP (2 mM) is monitored by the time-dependent decrease in DAPI-induced fluorescence. In the presence of OP, the rate of hydrolysis increases as the Cu^{2+} concentration in the reaction mixture rises from 10^{-8} to 10^{-5} M. The rate differs for each nucleic acid template used; hydrolysis of poly(dA-dT) > denatured DNA > double-stranded DNA. However, millimolar amounts of OP do not hydrolyze the template even in the presence of Cu^{2+} (10^{-6} M) when DNA is complexed with either *Escherichia coli* DNA polymerase I or *Euglena gracilis* or wheat germ RNA polymerase II. Under the same conditions, OP inhibits the activity of both varieties of RNA polymerase II with pK_i's of 3.4 and 3.0, respectively. The addition of neocuproine from 10^{-5} to 10^{-3} M to chelate any Cu^{2+} present in the reaction mixture does not change this inhibition. In contrast, OP does not affect DNA polymerase I activity. Thus, OP inhibits enzyme activity of a complex of RNA polymerase with nucleic acid template by chelation of metal atoms essential for the function of these polymerases rather than by hydrolysis of their template. Zinc is the only enzymatically active metal associated with RNA polymerases. This functional role is confirmed further by the demonstration that these enzymes are also inhibited by other chelating agents whose structures differ distinctively from that of OP: dipicolinic acid, 8-hydroxyquinoline, α,α′-bipyridyl, and 8-hydroxyquinoline-5-sulfonic acid.

The chelating agent 1,10-phenanthroline (OP) has frequently been used to study the functional role of zinc in enzymes (Chang & Bollum, 1970; Scrutton et al., 1971; Auld et al., 1974; Slater et al., 1972; Springgate et al., 1973). It has a particularly high affinity for zinc, and its high solubility permits the use of concentrated solutions (Sillen & Martell, 1964). Further, its relatively lower affinity for Mg^{2+}, a metal required for activation of all the nucleotidyl transferases (Mildvan, 1979), makes it particularly suitable for studies of this class of enzymes. The occurrence of OP inhibition of enzyme activity generally leads to the inference that chelation of a functional metal is the underlying mechanism. This assumption is usually tested by using nonchelating analogues of OP which are known not to inhibit activity.

However, recent reports have provided clear evidence that this generalization is not valid. Thus, in the presence of thiols, micromolar quantities of OP form complexes with Cu^{2+} which hydrolyze DNA (Marshall et al., 1982). OP, in millimolar amounts, also forms complexes with the Mg primer essential for DNA polymerase activity (Abraham & Modak, 1983).

† This work was supported, in part, by Grant-in-Aid GM24989 from the National Institutes of Health of the Department of Health, Education and Welfare.

* Address correspondence to this author at the Center for Biochemical and Biophysical Sciences and Medicine.

0006-2960/86/0425-2941$01.50/0