Maley, F., & Guarino, D. V. (1977) *Biochem. Biophys. Res. Commun. 77*, 1425-1430.
Markert, C., & Møller, F. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 753-763.
Markert, C. L., & Massaro, E. J. (1968) *Science (Washington, D.C.) 162*, 695.
McLoughlin, D. J., Shahied, I. I., & MacQuarrie, R. (1978) *Arch. Biochem. Biophys. 188*, 78-82.
Mukerjee, P., & Mysels, K. J. (1971) *Critical Micelle Concentrations of Aqueous Surfactant Systems*, NSRDS-NBS36 Superintendant of Documents, U.S. Government Printing Office, Washington, DC.
Nakaya, K., Yamada, K., Onozawa, Y., & Nakamura, Y. (1971) *Biochim. Biophys. Acta 251*, 7-13.
Nelson, C. A. A. (1971) *J. Biol. Chem. 246*, 3895-3901.
Nisselbaum, J. S., & Bodansky, O. (1963) *J. Biol. Chem. 238*, 969-974.
Novoa, W. B., & Schwert, G. W. (1961) *J. Biol. Chem. 236*, 2150-2153.
Nozaki, Y., Reynolds, J. A., & Tanford, C. (1974) *J. Biol. Chem. 249*, 4452-4459.
Osipow, L. I. (1962) *Surface Chemistry*, p 18, Reinhold, New York.
Reynolds, J. A., & Tanford, C. (1970a) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1002-1007.
Reynolds, J. A., & Tanford, C. (1970b) *J. Biol. Chem. 245*, 5161-5165.
Saraswat, H. C. (1953) *Proc.—Indian Acad. Sci., Sect. A 38A*, 220.
Satake, I., & Yang, J. T. (1976) *Biopolymers 15*, 2263-2275.
Stauffer, C. E., & Treptow, R. S. (1973) *Biochim. Biophys. Acta 295*, 457-466.
Steinhardt, J. (1975) *Protein-Ligand Interact., Proc. Symp.*, 412-426.
Steinhardt, J., & Reynolds, J. A. (1969) *Multiple Equilibria in Proteins*, Academic Press, New York.
Steinhardt, J., Scott, J. R., & Birdi, K. S. (1977) *Biochemistry 16*, 718-725.
Tanaka, H., Horiachi, Y., & Konishi, K. (1973) *Anal. Biochem. 66*, 489-491.
Tanford, C. (1973) *The Hydrophobic Effect*, Wiley, New York.
Waehneldt, T. V. (1975) *BioSystems 6*, 176.
Weber, K., & Kuter, D. J. (1971) *J. Biol. Chem. 246*, 4505-4509.
Wieland, T. H., & Pfleiderer, G. (1957) *Biochem. Z. 329*, 112-116.
Wilkinson, J. H. (1970) *Isoenzymes*, 2nd ed., Lippincott, Philadelphia.
Wojtczak, L., & Nalecz, M. H. (1979) *Eur. J. Biochem. 94*, 99-107.
Wroblewski, F., & Gregory, K. F. (1961) *Ann. N.Y. Acad. Sci. 94*, 912.

Inactivation of RTEM β-Lactamase from *Escherichia coli* by Clavulanic Acid and 9-Deoxyclavulanic Acid†

Robert L. Charnas and Jeremy R. Knowles*

ABSTRACT: The interaction of the TEM-2 β-lactamase with 9-deoxyclavulanic acid (**3**) and with both extensively labeled (**2**) and specifically labeled (**1**) clavulanic acid has been studied. The close similarity between 9-deoxyclavulanate and clavulanate in kinetic, spectroscopic, and protein chemical terms shows that the allyl alcohol group of clavulanate is irrelevant to its action as a β-lactamase inactivator. Use of the radiolabeled samples of clavulanate shows that, of three irreversibly inactivated forms of the enzyme, two contain the whole clavulanate skeleton and the third only retains the carbon atoms of the original β-lactam ring. These findings allow the complex interaction between clavulanic acid and the β-lactamase to be defined more narrowly in chemical terms.

β-Lactam antibiotics comprise one of the major classes of antibacterial agents, exerting their lethal effects by interfering with the metabolic machinery responsible for the normal growth and development of the bacterial cell wall (Blumberg & Strominger, 1974; Tomasz, 1979). Bacterial resistance to these lethal effects poses an obvious clinical problem, and this resistance has a number of different origins. The most prevalent cause of resistance derives from the production by the bacterium of a β-lactamase, which catalyzes the hydrolysis of the β-lactam in penicillins and cephalosporins to yield the innocuous penicilloic or cephalosporoic acids. Although the β-lactamase presents a ready target for the design of a drug that would overcome the defense mechanism of the bacterium, the problem was originally attacked by the development of β-lactam antibiotics that were less susceptible to the action of the β-lactamase. This situation changed with the discovery of clavulanic acid (Brown et al., 1976), a potent inhibitor and inactivator of β-lactamases. The announcement of clavulanic acid in 1976 heralded the discovery and synthesis of a number of other β-lactamase inactivators (English et al., 1978; Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979; Cartwright & Coulson, 1979; Gordon et al., 1980; Fisher & Knowles, 1980), as well as the more intense scrutiny of the mechanism of action of the enzyme. These studies have not only clarified the normal pathway of β-lactam hydrolysis by the enzyme but have also elucidated some of the details of the reactions that lead to enzyme inactivation.

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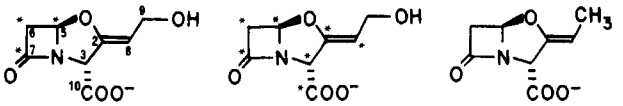
The interaction of clavulanic acid with the TEM-2 β-lactamase from *Escherichia coli* has been studied [Fisher et al., 1978; Charnas et al., 1978; see also Durkin & Viswanatha (1978), Labia & Peduzzi (1978), Cartwright & Coulson (1979) and Reading & Hepburn (1979)], and it is evident that clavulanic acid displays the most complex behavior of all the β-lactamase inactivators so far investigated. From our earlier work, clavulanic acid acts in three ways: as a substrate for the β-lactamase, as a slow transient inhibitor of the enzyme, and as an irreversible inactivator. There are three forms of irreversibly inactivated enzyme that can be discerned by isoelectric focusing, one of which may be reactivated (apparently to the native enzyme) by treatment with hydroxylamine. Although some spectroscopic information on the inactive enzyme was obtained (Charnas et al., 1978), the multiplicity of inactive species made it difficult to propose chemical structures for all the various enzyme complexes produced in the reaction with clavulanic acid. To elucidate some of these problems, we have investigated the interaction of the TEM-2 β-lactamase with some chemically modified and radiolabeled clavulanic acid derivatives (kindly made available to us by the Beecham Group). The results of these studies provide some helpful constraints on the range of mechanistic postulates for the interaction of this important metabolite with the β-lactamase.

Experimental Procedures

Materials

β-Lactamase was isolated from *E. coli* W3310 carrying the RP4 plasmid (Matthews & Hedges, 1976). The enzyme is a TEM-2 β-lactamase (Sykes & Matthews, 1976) and had a specific catalytic activity of 4000 units/A_{280nm} (enzyme absorption). A unit of enzyme activity is defined as that amount which will catalyze the hydrolysis of 1 μmol of benzylpenicillin/min at 30 °C, pH 7.0. The enzyme was purified as described previously (Fisher et al., 1978) and was homogeneous (>95%) as judged by isoelectric focusing in polyacrylamide gels in the presence of 4 M urea.

Clavulanic Acid Derivatives. [5,6,7-$^{14}C_3$]Clavulanic acid (**1**), [2,3,5,6,7,8,10-$^{14}C_7$]clavulanic acid (**2**), 9-deoxyclavulanic



1 **2** **3**

acid (**3**), and benzyl clavulanate were generous gifts from Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey, United Kingdom. [5,6,7-$^{14}C_3$]Clavulanic acid [see Elson & Oliver (1978)] was supplied as cream-colored crystals of the sodium salt tetrahydrate and had a specific radioactivity of 0.19 mCi/mmol. The three β-lactam ring carbon atoms contained 95% of the radioactivity. [2,3,5,6,7,8,10-$^{14}C_7$]-Clavulanic acid [see Elson & Oliver (1978)] was supplied as cream-colored crystals of the sodium salt tetrahydrate and had a specific radioactivity of 1.43 mCi/mmol. The three β-lactam ring carbon atoms contained 39% of the radioactivity. The percentage of radioactivity in the three β-lactam carbons of each sample had been determined by the dibenzylamine degradation method (Stirling & Elson, 1979). 9-Deoxyclavulanic acid, as the sodium salt, was a cream colored powder. Benzyl clavulanate was supplied as a viscous pale yellow oil. All materials were stored desiccated at −20 °C.

Biosolve BB5-3 scintillation solubilizer was purchased from Beckman Instruments, Inc., Fullerton, CA, Liquiflour from New England Nuclear Co., Boston, MA, and toluene (scintillation grade) from Fisher Scientific.

9-Deoxyclavulanic acid was prepared by the method of Howarth (T. T. Howarth, private communication). Benzyl clavulanate (50 mg) in ethanol (2.5 mL) was mixed with an equal volume of an ethanolic suspension of 10% Pd/C (5 mg in 2.5 mL) under H_2 (1 atm) at room temperature. After 1 h, the atmosphere was changed to N_2, and the catalyst was removed by centrifugation (14000*g* for 5 min). A portion (1 mL) of the supernatant was evaporated to dryness under reduced pressure, and the residue was dissolved in ethanol (500 μL). To this solution excess ethereal diazomethane was added, and the product, methyl 9-deoxyclavulanate was purified by thin-layer chromatography on silica gel (eluant, ethyl acetate–hexane, 1:2, R_f 0.40). The remaining portion of supernatant from the reduction was neutralized with aqueous $NaHCO_3$ (1 M) and then subjected to column chromatography on silica gel G (Merck) eluting with 1-butanol–ethanol–water (4:1:1.75 by volume). The major product cochromatographed on silica gel plates with authentic 9-deoxyclavulanate (eluant, 1-butanol–2-propanol–water, 7:7:6 by volume; R_f 0.70).

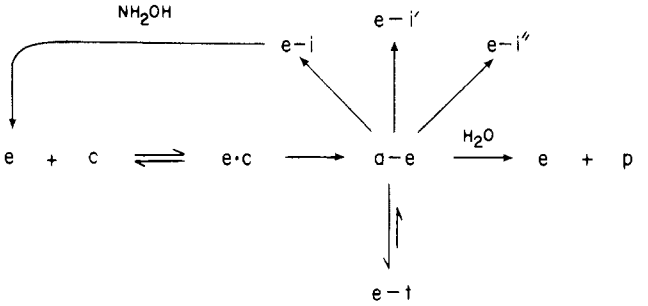
For the preparation of 9-deoxy[9-^{2}H]clavulanic acid, the Pd/C catalyst was washed twice with [1-^{2}H]ethanol and then dried under vacuum before activation with [^{2}H]hydrogen for 1 h. The solvent used was [1-^{2}H]ethanol.

For the preparation of 9-deoxy[9-^{3}H]clavulanic acid, the catalyst was activated with [^{3}H]hydrogen (~22 mCi/mmol), prepared by the slow addition of dioxane (90 μL) containing tritiated water (10 μL, 90 mCi/mmol) to a stirred solution of $LiAlH_4$ (1.0 M) in ethylene glycol dimethyl ether (200 μL). After isolation and purification, the 9-deoxy[9-^{3}H]clavulanate was >98% chemically pure, >90% radiochemically pure (thin-layer chromatography), and had a specific radioactivity of 0.36 mCi/mmol. The sample of methyl 9-deoxyclavulanate gave the following spectral data: IR (film) ν 2960, 2860, 1800, 1750, 1700, 1435, 1310, 870, 790, 730 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.62 (dd, 3 H, J = 1.5 and 6.8 Hz), 3.00 (d, 1 H, J = 16.5 Hz), 3.45 (dd, 1 H, J = 16.5 and 2.7 Hz), 3.77 (s, 3 H), 4.61 (dq, 1 H, J = 6.8 and 1.3 Hz), 4.98 (br d, 1 H, J = 1.4 Hz), and 5.64 (d, 1 H, J = 2.6 Hz); mass spectrum, m/z 197, 169, 141, 137, 113, 96, 71. The sample of methyl 9-deoxy-[9-^{2}H]clavulanate[2] gave the following spectral data: IR (film) ν 2960, 2850, 1800, 1750, 1690, 1435, 1300, 870, 730 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.62 (br s, 2 H), 3.00 (d, 1 H, J = 16.5 Hz), 3.45 (dd, 1 H, J = 16.5 and 2.7 Hz), 3.77 (s, 3 H), 4.98 (s, 1 H), and 5.64 (d, 1 H, J = 2.6 Hz); mass spectrum, m/z 199, 171, 141, 138, 113, 98, 71.

Methods

Solutions of clavulanic acid derivatives were prepared by weight with either a Cahn 25 electrobalance or a Roller-Smith microbalance. All ultraviolet measurements were made in 10 mM sodium phosphate buffer, pH 7.0, or in 10 mM *N*-ethylmorpholine acetate buffer, pH 7.0, at 30 °C. Enzyme activity was assayed by measuring the change in absorbance at 240 nm that accompanies the hydrolysis of the β-lactam ring of benzylpenicillin (Samuni, 1975). Amino acid analyses were performed on a Beckman 120B analyzer. Protein concentrations of inactive β-lactamase were determined either by amino acid analysis using native β-lactamase as the protein standard or by the method of Bradford (1976) using inactive β-lactamase as the protein standard. A value of $A_{280nm}^{0.1\%}$ of 1.0 was assumed. Protein hydrolyses were performed in 5.7 N HCl containing 0.1% (w/v) phenol at 110 °C in vacuo for 24 h. The results from two hydrolysates were averaged for

Scheme I: Minimal Scheme for Interaction of Clavulanic Acid with TEM-2 β-Lactamase[a]



[a] e, free enzyme; c, clavulanic acid; e·c, Michaelis complex of e and c; a-e, first-formed acyl-enzyme intermediate; e-t, transiently inhibited enzyme; p, products from hydrolytic breakdown of clavulanic acid; e-i, inactivated form of the enzyme that can be reactivated by hydroxylamine; e-i' and e-i'', inactivated forms of the enzyme that are inert to hydroxylamine.

each determination. Radioactivity measurements were made on a Beckman LS233 scintillation counter, using a scintillation cocktail composed of Biosolve–Liquifluor–toluene (10:5:85 by volume).

Carrier ampholytes for isoelectric focusing experiments were prepared by heating an aqueous solution (175 mL) of tetraethylenepentamine (30 mL) and itaconic acid (40 g) under reflux for 16 h. The resulting mixture was subjected to electrofocusing at 4 °C on a flat bed (20 × 40 cm) of Sephadex G-15 (10% v/v, total volume 250 mL), at 500 V for 72 h. The ampholytes focusing between pH 5 and pH 6 were eluted from the gel and then dialyzed through cellulose tubing (Spectrapor No. 2). Isoelectric focusing in polyacrylamide gels was done at 4 °C following the procedure of Wrigley (1971), using solutions of *N*-ethylmorpholine (150 mM) and acetic acid (150 mM) as the cathodic and anodic solutions, respectively.

Assays of enzyme inactivation were done as follows. A portion (50 μL) of a solution of β-lactamase (1 μM) was mixed with a buffered solution of clavulanic acid (14.95 mL, 0.2–25 μM) and incubated at 30 °C in a silanized flask. At appropriate intervals, samples (1.5 mL) were taken and mixed with a buffered solution (1.5 mL) of benzylpenicillin (2.8 mM) in an optical cuvette. The rate of hydrolysis of benzylpenicillin after 1.5 min indicated the amount of active enzyme. The number of hydrolytic turnovers before inactivation and the rate of decomposition of the transiently stable complex were determined as described previously (Fisher et al., 1978).

Inactivated β-lactamase was prepared as follows. Portions (500 μL) of a buffered solution of β-lactamase (78 μM) and portions (2.0 mL) of a buffered solution of clavulanic acid (20 mM) were equilibrated at 4, 30, or 43 °C and then mixed. After incubation at the appropriate temperature for 90 min, more than 96% of the catalytic activity of the enzyme had been lost. The protein was subjected to gel filtration on Sephadex G-50 in 25 mM *N*-ethylmorpholinium acetate, pH 7.0, and then dialyzed exhaustively at 4 °C against 0.1 M sodium phosphate buffer, pH 7.0.

Partial reactivation of inactive enzyme by hydroxylamine (Charnas et al., 1978) was achieved by treatment of the protein with hydroxylamine (14 mM) in 0.1 M sodium phosphate buffer, pH 7.0, at 30 °C for 30 min. Samples were then cooled to 4 °C before immediate isoelectric focusing.

Results and Discussion

The kinetic and chemical complexity of the interaction of clavulanic acid with the TEM-2 β-lactamase which was evident from our earlier studies (Fisher et al., 1978; Charnas et al., 1978) appears to require the formulation of a number of routes to inactive enzyme. The minimal scheme consistent with the kinetic results and the existence of three separable species of inactivated enzyme is shown in Scheme I, and it was the purpose of the present work to delineate some of the chemical events that give rise to the behavior required by this scheme.

Scheme II: Alternative Modes of Collapse of Tetrahedral Intermediate Formed by Attack of β-Lactamase on the β-Lactam Ring of Clavulanic Acid

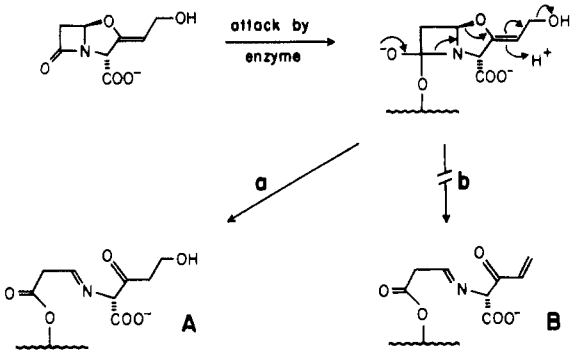


Table I: Kinetic Features of Inactivation of 9-Deoxyclavulanic Acid (3) and Clavulanic Acid with TEM-2 β-Lactamase

	9-deoxyclavulanic acid (3)	clavulanic acid
no. of hydrolytic turnovers before inactivation	30	115
K_{inact}[a] (μM)	3 ± 1	0.7 ± 0.3
k_{inact}[a] (s^{-1})	(2.2 ± 0.4) × 10^{-2}	(4.0 ± 0.3) × 10^{-2}
k_{react}[b] (s^{-1})	(2.7 ± 0.8) × 10^{-3}	(3.8 ± 0.3) × 10^{-3}

[a] Determined from a double-reciprocal plot of the initial rate of inactivation of the enzyme at different initial inactivator concentrations. [b] This is the rate constant for the decomposition of the transiently inhibited enzyme [see Fisher et al. (1978)].

Acyl-Enzyme. Consistent with the recent demonstration that the hydrolytic reaction catalyzed by the β-lactamase proceeds via an acyl-enzyme (Fisher et al., 1980) and with evidence from studies of other β-lactamase inactivating reagents [particularly 6β-bromopenicillanic acid (Knott-Hunziker et al., 1980; Cohen & Pratt, 1980) and penicillanic acid sulfone (Brenner & Knowles, 1981; Kemal & Knowles, 1981)], it seems very likely that clavulanic acid first acylates the enzyme. In the case of penicillanic acid sulfone, the evidence suggests that the first-formed tetrahedral intermediate yields the imine in which both rings of the penam have been cleaved (either synchronously or via the penicilloyl-enzyme; Brenner & Knowles, 1981; Kemal & Knowles, 1981), and an analogous formulation is certainly attractive for clavulanic acid. In this case, however, two types of collapse are possible, and these are shown in Scheme II. In pathway a the β-hydroxy ketone A is formed, and in pathway b the 9-hydroxy group is lost by elimination to give B. This latter path has been proposed (Labia & Peduzzi, 1978) and has the attraction of providing a Michael acceptor to accommodate subsequent enzyme inactivation. To distinguish between these alternatives, we studied the behavior of 9-deoxyclavulanic acid (3). This analogue cannot follow pathway b but will behave like clavulanic acid itself if pathway a is the preferred path. In Table I, the results of a kinetic investigation of 9-deoxyclavulanic acid are compared with those of clavulanic acid, and it is evident that the two compounds interact very similarly with the β-lactamase. This conclusion is reinforced by the following facts: (a) enzyme that has been inactivated by the 9-deoxy compound shows a 1.4-fold increase in A_{280nm} (compare 1.4-

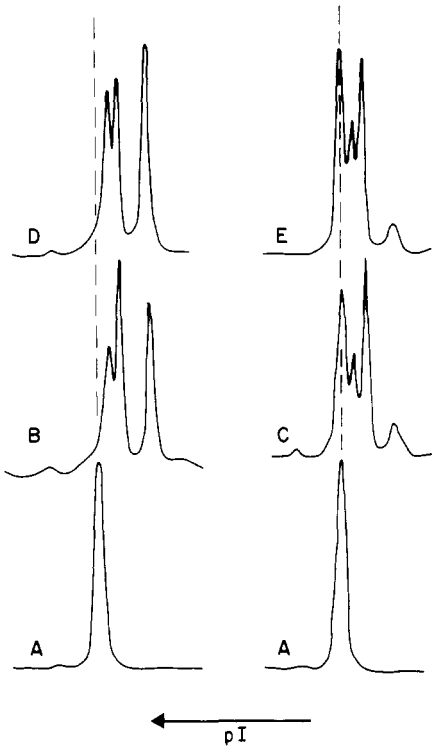
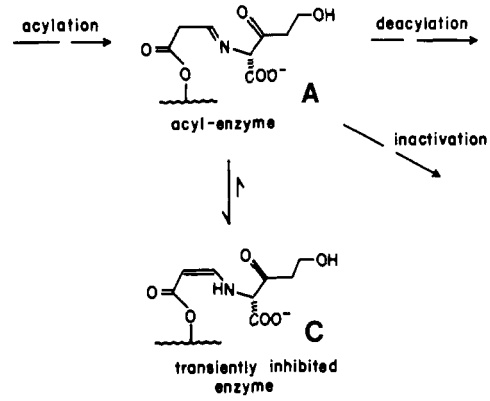


FIGURE 1: Densitometer scans of polyacrylamide isoelectric focusing gels. (A) Native β-lactamase; (B) enzyme after inactivation by excess clavulanic acid; (C) sample B after partial reactivation with hydroxylamine; (D) enzyme after inactivation by excess 9-deoxyclavulanic acid; (E) sample D after partial reactivation with hydroxylamine.

fold with clavulanate), (b) treatment of the inactivated enzyme with hydroxylamine restores ~25% of the catalytic activity (compare ~36% with clavulanate), and (c) isoelectric focusing of the inactivated enzyme shows three inactive protein species only one of which reacts with hydroxylamine to regenerate native enzyme (see Figure 1). These phenomena are all very similar to what is observed with clavulanic acid itself, and pathway b (Scheme II) is therefore unlikely to be followed by clavulanate. The allyl alcohol group of clavulanic acid must be irrevelant to its interaction with the isolated enzyme. The unimportance of the alcohol group evidently also extends to the action of clavulanate in vivo, since the Beecham group have demonstrated that 9-deoxyclavulanate and clavulanate have similar synergistic antibacterial potencies (Howarth et al., 1977).

Transiently Inhibited Enzyme. Since it appears that the first formed acyl-enzyme is the imine A (Scheme II), we may now ask what the structure of the transiently inhibited form of the enzyme (e-t in Scheme I) is. The most notable feature of the transiently inhibited enzyme is that it is chromophoric (Charnas et al., 1978), showing a strong absorption near 280 nm that has been assigned to a β-aminoacrylate group on the enzyme. This grouping seems to be characteristic of both the transiently inhibited enzyme and the irreversibly inactivated enzyme (Charnas et al., 1978) and is formed analogously when 6-chloropenicillanic acid sulfone (Cartwright & Coulson, 1979) and penicillanic acid sulfone (Brenner & Knowles, 1981; Kemal & Knowles, 1981) interact with the β-lactamase. The formation of e-t from the acyl-enzyme therefore involves the tautomerization of the imine A to the more stable chromophoric enamine C, as shown in Scheme III. To obtain support for this formulation, we used the ^{14}C-labeled clavulanate sample (**2**) to see if the transiently inhibited enzyme contains the whole clavulanate molecule bound to the protein, as is

Scheme III: Formation of Acyl-Enzyme from Clavulanic Acid and β-Lactamase and Its Conversion to the Transiently Inhibited Form of the Enzyme



required by Scheme III. Accordingly, β-lactamase was incubated at 30 °C with a 60-fold molar excess of **2** for 100 s and then rapidly cooled to 4 °C, at which temperature it was subjected to rapid gel filtration. The resulting protein fraction contained 30% native enzyme (as determined from the *initial* rate of benzylpenicillin hydrolysis), 50% of the transiently inhibited enzyme (as determined from the difference between the initial and final rates of benzylpenicillin hydrolysis as the sample was allowed to recover its catalytic activity at 30 °C), and 20% irreversibly inactivated enzyme (as determined from the ultimate loss of catalytic activity after reincubation at 30 °C). From the specific radioactivity of this protein fraction, 0.57 ± 0.03 mol of clavulanate was attached to the protein, of which 0.13 ± 0.03 mol derived from the irreversibly inactivated enzyme.[1] A total of 50% of transiently inhibited enzyme therefore contained 0.44 (0.57–0.13) mol of bound clavulanate. This experiment demonstrates that within experimental error, the transiently inhibited enzyme contains the *whole* clavulanate molecule attached to the protein. These data are therefore entirely in accord with the formulation of Scheme III.

Irreversibly Inactivated Enzyme. When the β-lactamase was completely irreversibly inactivated at 4 °C by a large (>150-fold) molar excess of the extensively labeled [^{14}C]-clavulanate sample (**2**) the isolated protein was found to contain 0.45 ± 0.05 mol of clavulanate/mol of enzyme. The label is covalently attached to the protein, and no radioactivity is lost on incubation in 1% sodium dodecyl sulfate and subsequent gel filtration or exhaustive dialysis. The fact that this value is substoichiometric, coupled with the knowledge that there are three species of inactive enzyme that can be separated by isoelectric focusing, made us suspect that one or more of the inactive species might have lost some portion of the clavulanate skeleton. This suspicion was reinforced by the finding that when the enzyme was inactivated at 4 °C with the *specifically* labeled clavulanate sample (**1**) the inactivated protein retained 0.82 ± 0.08 mol of label/mol of enzyme.

The most direct test of the possibility that one or more of the inactive species contains less than the whole clavulanate skeleton attached to the protein would be to isolate each of the inactive species. Unfortunately, all efforts to effect this separation on a preparative scale were unsuccessful. Neither ion-exchange chromatography nor electrophoresis in poly-

[1] This value was determined by subjecting the protein, after reincubation at 30 °C to allow the transiently inhibited enzyme to regain activity, to gel filtration to remove unattached radioactivity. The reactivated sample (which had 80% of the original catalytic activity) contained 0.13 ± 0.03 mol of clavulanate/mol of enzyme.

Table II: Temperature Dependence of Inactivation of Clavulanic Acid and 9-Deoxyclavulanic Acid with TEM-2 β-Lactamase

	temp of inactivation (°C)		
	4	30	43
Clavulanic Acid			
fraction of inactivated enzyme species:[a]			
e-i	0.25	0.34	0.61
e-i′	0.67	0.41	0.21
e-i″	0.08	0.25	0.17
increase in ϵ_{280nm} (M^{-1} cm^{-1})[b]	7000	10000	18000
fraction of catalytic activity restored by hydroxylamine	0.27	0.34	0.48[c]
labeling stoichiometry with **2** (mol/mol)	0.45 ± 0.05	0.68 ± 0.05	0.91 ± 0.05
labeling stoichiometry with **1** (mol/mol)	0.82 ± 0.08	0.82 ± 0.08	0.86 ± 0.09
9-Deoxyclavulanic Acid			
fraction of inactivated enzyme species:[a]			
e-i	0.13	0.18	0.24
e-i′	0.71	0.57	0.28
e-i″	0.16	0.24	0.48
increase in ϵ_{280nm} (M^{-1} cm^{-1})[b]	*d*	10000	*d*
labeling stoichiometry with deoxy[^{3}H]clavulanate[e]	0.18 ± 0.04	0.26 ± 0.05	0.53 ± 0.10

[a] Determined by densitometer scans of the gels stained with Coomassie Blue. The percentages have an average error of ±0.04. [b] Based on a molar extinction coefficient of 29 400 M^{-1} cm^{-1} for the native enzyme. [c] The value of 0.48 is lower than expected (0.61) due to some thermal denaturation of the protein at this temperature. [d] Not determined. [e] The errors in these values are precision estimates only; see footnote 2 of the text.

acrylamide or Sephadex gels produced an analytical separation, and the one method (isoelectric focusing) that gave good separations at the analytical level was not susceptible to scale-up. The unacceptably low recovery of the three inactive species from preparative isoelectric focusing gels precluded any precise estimate of the specific radioactivities of the individual species. We found, however, that the relative proportions of the three inactive species depend markedly on the temperature at which the inactivation is performed. Whereas at 30 °C the three forms are in roughly equal amounts, if the inactivation is done at 4 °C, the middle band constitutes ~67% of the total, and if the inactivation is done at 43 °C, the band of lowest p*I* predominates (at ~61% of the total; see Table II). By measurement therefore, of the relative proportions of the three forms (from densitometer scans of the stained isoelectric focusing gels) and the overall labeling stoichiometries, at three different temperatures, approximate estimates of the labeling stoichiometries for each species may be obtained. In Table II are listed the data required for this analysis, providing three simultaneous equations (one at each temperature)

$$\text{overall stoichiometry} = \chi(\text{e-i}) + \chi'(\text{e-i}') + \chi''(\text{e-i}'')$$

where χ, χ', and χ'' are the individual stoichiometries of the three inactive forms of the enzyme. The values of χ, χ', and χ'' are obtained by using Cramer's rule (Thomas, 1968). The errors in such an analysis are, of course, very high, but the results provide important clues as to the nature of the inactive forms of the enzyme.

From the results of the extensively labeled clavulanic acid (**2**) the stoichiometries of labeling of e-i, e-i′, and e-i″ (in order of decreasing p*I*) are 0.9 ± 0.3, 0.1 ± 0.3, and 1.2 ± 0.3 mol/mol of protein, respectively. A significant portion of the clavulanate molecule has therefore been lost from e-i′, whereas e-i and e-i″ appear to retain the whole clavulanate skeleton. To discover which part of the inactivator molecule is lost, we performed analogous experiments with the specifically labeled clavulanic acid (**1**). In this case, the results indicate that the extents of labeling of e-i, e-i′, and e-i″ are 0.7 ± 0.3, 0.9 ± 0.3, and 0.9 ± 0.3 mol/mol of protein, respectively. These results suggest that in *each* of the inactive forms of the enzyme, the carbon atoms that constituted the original β-lactam ring are retained. We may conclude, therefore, that e-i and e-i″ retain the whole clavulanate molecule, while in e-i′ only the carbon atoms of the original β-lactam ring remain bound. The necessarily large error limits on the derived stoichiometries do not allow a decision between, for instance, a transimination [such as that proposed elsewhere (Brenner & Knowles, 1981; Kemal & Knowles, 1981)] or a cleavage of the bond between C-5 and C-6 (Hammarström & Strominger, 1975; Marquet et al., 1979) (this fragmentation would only leave two of the three labeled carbons of **1** attached, but the error limits on the experimental results allow this possibility), but it is clear that some such process is occurring. This conclusion is supported by the overall stoichiometries observed at the three temperatures when 9-deoxy[9-^{3}H]clavulanic acid is used as the inactivator (Table II). Qualitatively, the amount of covalently bound radioactivity increases with the inactivation temperature, as the proportion of the fragmented species, e-i′, falls.[2]

Finally, further information on the nature of the three inactive forms of the enzyme may be gleaned from the changes in A_{280nm}. As is shown in Table II, the observed rise in A_{280nm} of the enzyme on inactivation increases as the inactivation temperature increases, implying that it is e-i (and possibly e-i″, from the data on the 9-deoxy compound) that is the chromophoric species. This is confirmed by the fact that hydroxylamine, which selectively converts e-i to active enzyme (Figure 1), largely eliminates the new chromophore at 280 nm.

In summary, we have three forms of inactive enzyme having the following properties. The first species, e-i, is chromophoric at 280 nm, contains the whole clavulanate molecule attached to the enzyme, and reacts completely with hydroxylamine to regenerate active enzyme. The second, e-i′, has no strong chromophore at 280 nm and has lost more than half the clavulanate skeleton. The third, e-i″, has the lowest p*I* and contains the whole clavulanate molecule. While it is not possible on the basis of these data to assign unique structures to each of the inactive enzyme species, the results provide helpful constraints on the multitude of possible paths.

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References

Blumberg, P. M., & Strominger, J. L. (1974) *Bacteriol. Rev.* *38*, 291–335.

[2] The deoxy[^{3}H]clavulanate was prepared analogously to the ^{2}H material, the NMR of which showed >80% incorporation of ^{2}H at *both* C-8 and C-9. Since the formation of the acyl-enzyme (A, Scheme III) will labilize the C-8 protons, the fact that the overall stoichiometries in Table II are less than the sum of e-i + e-i″ is not surprising.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Brenner, D. G., & Knowles, J. R. (1981) *Biochemistry* (in press).
Brown, A. G., Butterworth, D., Cole, M., Hanscomb, G., Hood, J. D., & Reading, C. (1976) *J. Antibiot. 29*, 668–669.
Cartwright, S. J., & Coulson, A. F. W. (1979) *Nature (London) 278*, 360–361.
Charnas, R. L., Fisher, J., & Knowles, J. R. (1978) *Biochemistry 17*, 2185–2189.
Cohen, S. A., & Pratt, R. F. (1980) *Biochemistry 19*, 3996–4003.
Durkin, J. P., & Viswanatha, T. (1978) *J. Antibiot. 31*, 1162–1169.
Elson, S. W., & Oliver, R. S. (1978) *J. Antibiot. 31*, 586–592.
English, A. R., Retsema, J. A., Girard, A. E., Lynch, J. E., & Barth, W. E. (1978) *Antimicrob. Agents Chemother. 14*, 414–419.
Fisher, J., & Knowles, J. R. (1980) in *Enzyme Inhibitors as Drugs* (Sandler, M., Ed.) pp 209–218, Macmillan, London.
Fisher, J., Charnas, R. L., & Knowles, J. R. (1978) *Biochemistry 17*, 2180–2184.
Fisher, J., Belasco, J. G., Khosla, S., & Knowles, J. R. (1980) *Biochemistry 19*, 2895–2901.
Gordon, E. M., Chang, H. W., Cimarusti, C. M., Toeplitz, B., & Gougoutas, J. Z. (1980) *J. Am. Chem. Soc. 102*, 1690–1702.
Hammarström, S., & Strominger, J. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3463–3467.
Howarth, T. T., Goodacre, J., & Ponsford, R. J. (1977) *Ger. Offen.* 2616088.
Kemal, C., & Knowles, J. R. (1981) *Biochemistry* (in press).
Knott-Hunziker, V., Orlek, B. S., Sammes, P. G., & Waley, S. G. (1979) *Biochem. J. 177*, 365–367.
Knott-Hunziker, V., Orlek, B. S., Sammes, P. G., & Waley, S. G. (1980) *Biochem. J. 187*, 797–802.
Labia, R., & Peduzzi, J. (1978) *Biochim. Biophys. Acta 526*, 572–579.
Marquet, A., Frère, J.-M., Ghuysen, J.-M., & Loffet, A. (1979) *Biochem. J. 177*, 909–916.
Matthew, M., & Hedges, R. W. (1976) *J. Bacteriol. 125*, 713–718.
Pratt, R. F., & Loosemore, M. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4145–4149.
Reading, C., & Hepburn, P. (1979) *Biochem. J. 179*, 67–76.
Samuni, A. (1975) *Anal. Biochem. 13*, 17–26.
Stirling, I., & Elson, S. W. (1979) *J. Antibiot. 32*, 1125–1129.
Sykes, R. B., & Matthews, M. (1976) *J. Antimicrob. Chemother. 2*, 115–117.
Thomas, G. B. (1968) *Calculus and Analytical Geometry*, 4th ed., Addison-Wesley, Reading, MA.
Tomasz, A. (1979) *Annu. Rev. Microbiol. 33*, 113–137.
Wrigley, C. W. (1971) *Methods Enzymol. 22*, 559–560.

Methotrexate and Folate Binding to Dihydrofolate Reductase. Separate Characterization of the Pteridine and *p*-Aminobenzoyl Binding Sites by Resonance Raman Spectroscopy†

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ABSTRACT: By using 324- and 350.6-nm excitation, it is possible to obtain selectively the resonance Raman spectra of the *p*-aminobenzoyl and pteridine chromophores, respectively, within methotrexate (MTX) or folate. Thus, for a single ligand, by changing the wavelength for excitation, the geometric conformations of both chromophores can be monitored separately. Resonance Raman spectra are reported for MTX bound to dihydrofolate reductases from *Escherichia coli* and from *Lactobacillus casei*, in each case in the presence and absence of NADPH. Additionally, some data are presented for enzyme-bound folate. The resonance Raman data support the conclusions of other workers that MTX binds with its pteridine ring protonated while the pteridine ring within folate is bound as a neutral species. However, for MTX, marked differences exist between the electronic distribution in the protonated pteridine ring for the ligand free in solution and for the bound species. The rearrangement of the pteridine electrons over and above that accompanying protonation explains the absorption properties of bound MTX, and together with protonation may account in part for the high affinity of MTX for the enzyme. The resonance Raman spectra show that slight differences exist between the pteridine sites for MTX in the three dihydrofolate reductases studied while no differences could be detected among the *p*-aminobenzoyl sites. In each MTX–protein complex, however, there appears to be a marked change in the geometry of the amide group in the benzoyl linkage of MTX compared to the geometry found in the free ligand. The resonance Raman spectra of MTX bound to the enzymes were unchanged upon the addition of the cofactor NADPH, indicating that the cofactor does not bring about marked electron rearrangement in the bound ligands.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-linked reduction of dihydrofolate to tetrahydrofolate. The inhibition of this enzyme by the drug methotrexate (MTX)[1] (Figure 1) is the source of methotrexate's therapeutic effectiveness in the treatment of childhood leukemia and of several other cancers. Although methotrexate bears an overall structural similarity to the substrates (Figure 1), the affinity of the inhibitor for

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[1] Abbreviations used: MTX, methotrexate; RR, resonance Raman; DHFR, dihydrofolate reductase; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

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