Chemical Studies on the Inactivation of *Escherichia coli* RTEM β-Lactamase by Clavulanic Acid†

Robert L. Charnas,‡ Jed Fisher,§ and Jeremy R. Knowles*

ABSTRACT: Incubation of clavulanic acid with the β-lactamase from *Escherichia coli* RTEM leads to enzyme-catalyzed depletion of clavulanic acid, to transient inhibition, and to irreversible inactivation of the enzyme. Both the transiently inhibited and the irreversibly inactivated species show a marked increase in the absorbance at 281 nm that is proportional to the decrease in enzyme activity. Hydroxylamine treatment of irreversibly inactivated enzyme restores about one-third of the catalytic activity, with a concomitant decrease in absorbance at 281 nm. Polyacrylamide isoelectric focusing of the irreversibly inactivated enzyme shows three bands of approximately equal intensity, different from native enzyme. Upon hydroxylamine treatment, one of the three bands disappears and now focuses identically with native enzyme. It is evident that the irreversible inactivation of enzyme by an excess of clavulanic acid generates three products, one of which can be reactivated by hydroxylamine.

Following the recent discovery of several new classes of naturally occurring β-lactams (Hashimoto et al., 1976; Howarth et al., 1976; Brown et al., 1977), we have undertaken a detailed investigation of the interaction of one of these, clavulanic acid (I), with the plasmid-encoded β-lactamase from *Escherichia coli* RTEM. In the previous paper, kinetic evidence has been presented that demonstrates that clavulanic acid (Howarth et al., 1976) interacts with the *E. coli* β-lactamase in three ways: the clavulanic acid is destroyed catalytically, and the enzyme is both transiently inhibited and irreversibly inactivated (Fisher et al., 1978). The present paper provides chemical and spectroscopic evidence on the nature of these processes.

Materials and Methods

Sodium clavulanate and β-lactamase were obtained as described by Fisher et al. (1978). Enzyme concentrations were determined spectrophotometrically using the literature value of 46.7 nmol/mL per unit of absorbance at 281 nm.

Deuterium oxide (>99.8%) was purchased from Bio-Rad Laboratories, Richmond, Calif.

Sodium boro[^{3}H]hydride of specific activity 325 mCi/mmol was purchased from New England Nuclear, Boston, Mass.

O-[^{14}C]*Methylhydroxylamine* was purchased from New England Nuclear as a solution of the hydrochloride salt in 70% aqueous ethanol. The specific activity was 10 mCi/mmol. Thin-layer chromatography showed the material to be greater than 98% radiochemically pure, and it was used without further purification.

Isolation of the Transient Complex. (Unless otherwise stated, all reactions were run in 0.1 M potassium phosphate buffer, pH 7.0.) Solutions of β-lactamase (150 μL of a 57 μM solution) and sodium clavulanate (150 μL of a 4 mM solution), preequilibrated at 30 °C, were mixed and incubated for 2 min at 30 °C. The reaction was quenched by cooling in ice for 3 min, and the mixture was then applied to a column (1.0 × 6.5 cm) of Sephadex G-25 at 4 °C. The column was eluted with 0.1 M potassium phosphate buffer, pH 7.0, at 30 mL/h. Fractions were stored in ice, assayed for activity, and then analyzed spectrophotometrically.

Preparation and Isolation of Inactivated Enzyme. Solutions of β-lactamase (57 μM) and sodium clavulanate (15 mM) in 0.1 M potassium phosphate buffer, pH 7.0, were mixed and incubated for 1.5 h at 30 °C. The residual activity was less than 2% that of the native enzyme; excess clavulanate was removed by exhaustive dialysis at 4 °C.

Hydroxylamine Reactivation. Aqueous hydroxylamine (20 μL of a 40 mM solution, pH 7.0) and inactivated enzyme solution (20 μL of a 57 μM solution, pH 7.0), preequilibrated at 37 °C, were mixed and incubated at 37 °C. Samples (2 μL) were withdrawn at various times and assayed for catalytic activity.

O-[^{14}C]*Methylhydroxylamine Reactivation.* Aqueous *O*-[^{14}C]methylhydroxylamine hydrochloride (50 μL of a 10 mM solution) was added to H_2SO_4 (5 μL of a 1 M solution) and subjected to bulb-to-bulb distillation under vacuum. Most (80–90%) of the radioactivity remained after distillation. The reactivation of the enzyme was initiated by the rapid successive addition of sodium phosphate buffer (5 μL of a 200 mM solution, pH 7.0), sodium hydroxide (10 μL of a 1 N solution), and inactivated enzyme solution (25 μL of a 5 μM solution). The incubation mixture was then tightly stoppered to prevent loss of the *O*-methylhydroxylamine and incubated at 37 °C for 24 h.

Isoelectric Focusing. Acrylamide and *N,N'*-methylenebis(acrylamide) were purchased from Bio-Rad; carrier ampholytes were purchased as a 40% aqueous solution from Brinkmann Instruments, Westbury, N.Y. All other reagents were the highest grade available commercially and were used without further purification. Disc gel isoelectrofocusing was run on a Bio-Rad Model 300 apparatus following the procedure of Wrigley (1971). Slab gels were run on Desaga Brinkmann double-chamber thin-layer electrophoresis apparatus according to the manufacturer's instructions. Gels were stained and destained according to Wrigley (1971); densitometer traces were obtained on a Gilford 240 spectrophotometer assembly.

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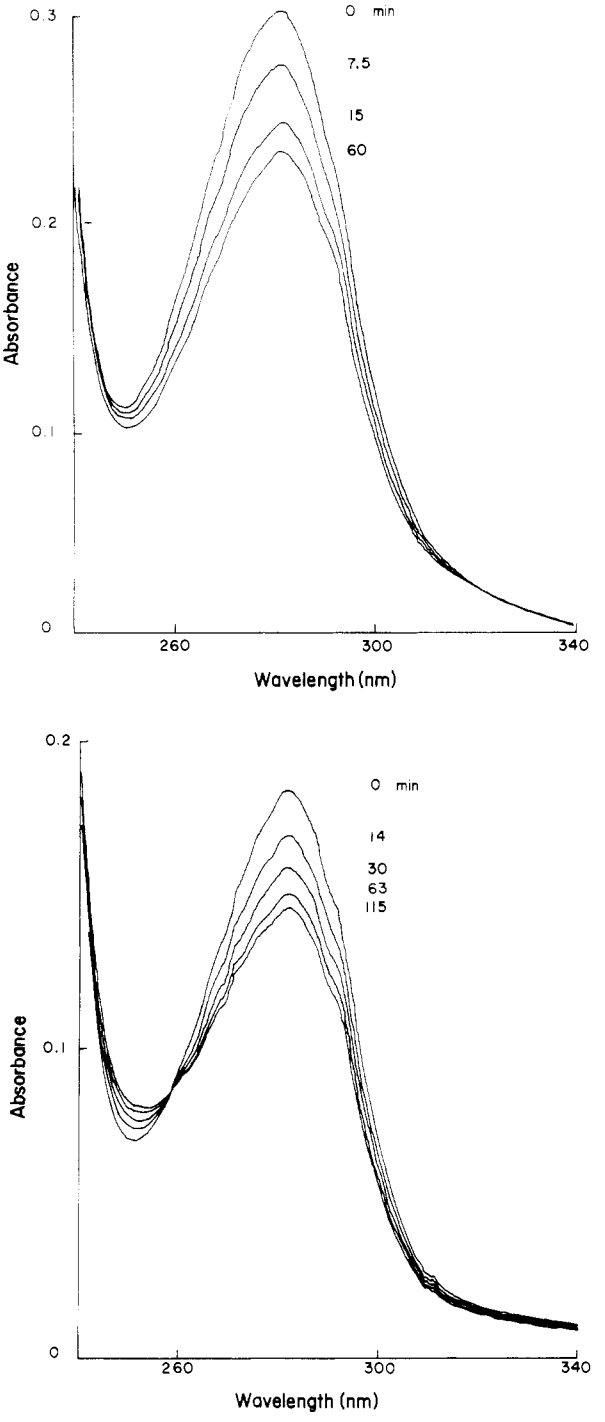


FIGURE 1: (Top) Spectral changes in the transiently inhibited complex between clavulanic acid and β-lactamase. The transient complex was isolated by gel filtration in the cold, and the spectral changes shown were monitored as the recovery reaction proceeded at 30 °C. For details, see the text. (Bottom) Spectral changes in β-lactamase after inactivation by clavulanic acid and treatment with hydroxylamine. Inactivated enzyme was mixed with hydroxylamine to give final concentrations of 26 μM and 5 mM, respectively. Spectra were recorded as the reaction proceeded at 30 °C. For details, see the text.

Infrared spectra were taken in 100 mM sodium phosphate buffer (pD 7.0) in deuterium oxide using 0.1-mm CaF_2 cells, on a Digilab FT-15 Fourier transform instrument. Spectra were recorded at 8 cm^{-1} resolution using the double beam ratio method. Sodium clavulanate and β-lactamase were prepared for infrared spectroscopy by repeated freeze-drying from deuterium oxide solution.

Results and Discussion

In the previous paper (Fisher et al., 1978), it was shown that there are three types of interaction between clavulanic acid and the β-lactamase from *E. coli* RTEM. First, the enzyme catalyzes the loss of enzyme-inhibitory activity of clavulanate, with the consequence that 115 molecules of inhibitor are required to inactivate one molecule of enzyme. Secondly, the enzyme is transiently inhibited by clavulanate, with rates of loss and recovery of enzyme activity that are incompatible with the mere formation of a Michaelis-type complex. Thirdly, the enzyme is irreversibly inactivated by clavulanic acid. The chemical and spectroscopic characteristics of these three processes are discussed in turn.

Enzyme-Catalyzed Hydrolysis of Clavulanic Acid. It seemed probable that the loss of enzyme-inhibitory capacity of clavulanic acid upon incubation with the enzyme was the simple result of enzyme-catalyzed hydrolysis of the β-lactam ring. Unfortunately, clavulanic acid possesses no usable chromophore in the visible or ultraviolet, and neither the iodometric assay for penicillins (Novick, 1962) nor the hydroxamate assay (Feigl, 1960) are suitable for quantitative determination. The problem is exacerbated by the expected instability of the molecule after the β-lactam ring has been hydrolyzed. However, clavulanic acid does possess the strong and characteristic β-lactam carbonyl absorption at 1780 cm^{-1} (D_2O).

Infrared spectra of clavulanic acid in D_2O before and after incubation with β-lactamase (in a molar ratio of 20:1, clavulanate:enzyme) clearly show the disappearance of the β-lactam carbonyl group at 1780 cm^{-1} on treatment with the enzyme. It is therefore probable that clavulanic acid is a substrate for the β-lactamase.

Inhibition and Inactivation of the Enzyme. The inhibition of the β-lactamase by clavulanate involves two major processes, a slowly reversible inhibition and an irreversible inactivation (Fisher et al. 1978), and a minimal kinetic mechanism has been proposed for these interactions. (See Scheme IB of Fisher et al., 1978.)

Ultraviolet Absorbance Changes. In order to obtain more direct evidence for the transient complex (e-t) of this scheme, β-lactamase was incubated at 30 °C with a 300-fold molar excess of clavulanic acid for 2 min. On cooling to 4 °C, rapidly isolating the enzyme by gel filtration at this temperature, and pooling all the enzyme fractions containing catalytic activity, approximately 15% of the enzyme's catalytic activity remained (compare Figure 1 of Fisher et al., 1978). The ultraviolet spectrum of this material is shown in Figure 1 (top), from which it is evident that there has been an increase in absorbance at 281 nm of about 1.5-fold. On warming the solution to 30 °C, the absorbance at 281 nm decreases to a limiting value that is about 1.1-fold higher than that of native enzyme. This decrease in absorbance occurs with a concomitant increase in catalytic activity ($t_{1/2} \sim 10$ min). The final value of the enzyme activity was 70%. In terms of the kinetic mechanism it is clear that, after the gel filtration, 15% of the enzyme was active, 30% was irreversibly inactivated, and 55% was therefore in the transiently inhibited form. Evidently the transiently inhibited form, e-t, had developed a strong chromophore at 281 nm, and the reaction that regenerates the catalytic activity destroys this chromophore. The fact that there is a residual increase in absorbance after the regeneration of e-t is complete indicates that the irreversibly inactivated enzyme (of which there is 30% at the end of the recovery reaction) also has a new chromophore. This is confirmed by what follows.

Irreversibly inactivated enzyme, e-i, was prepared by in-

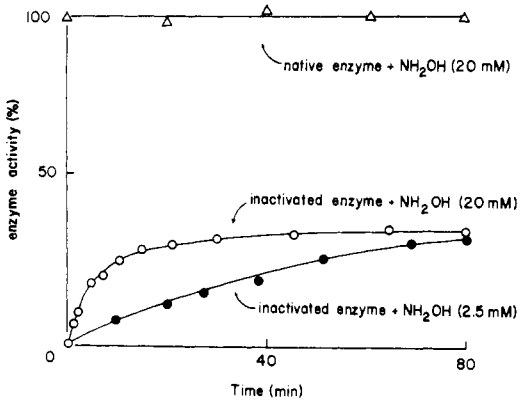


FIGURE 2: Partial reactivation of inactivated β-lactamase by hydroxylamine. The incubation was done at 30 °C. For details, see the text.

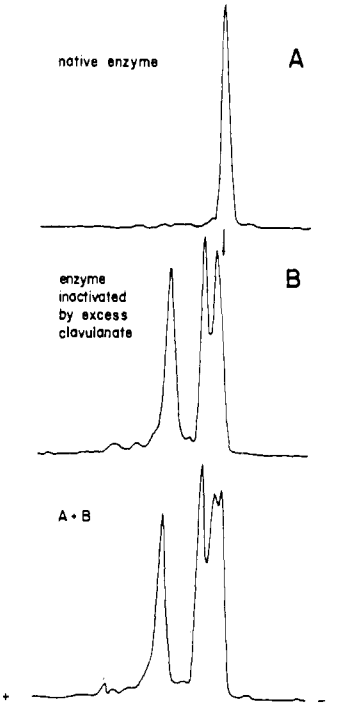
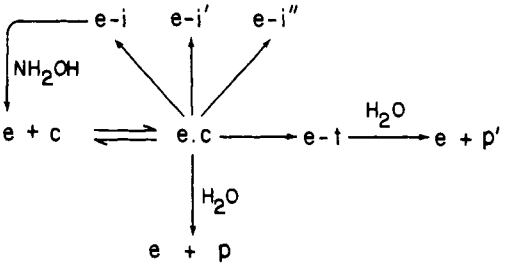


FIGURE 3: Densitometer scans of native and inactivated β-lactamase after polyacrylamide gel isoelectric focusing.

SCHEME I: Minimal Scheme for the Interaction of Clavulanic Acid with *E. coli* β-Lactamase.[a]



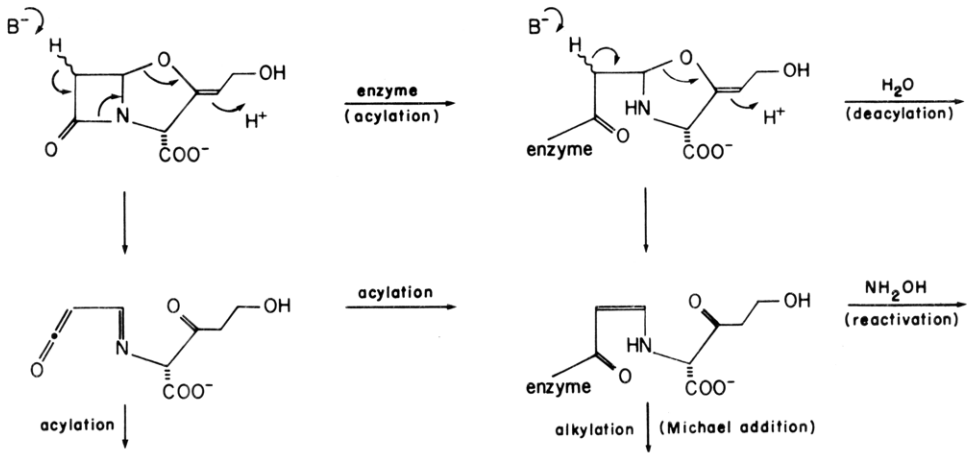
[a] e, free enzyme, c, clavulanic acid; e•c, the Michaelis–complex of e and c; p, the product from hydrolytic turnover; p′, the clavulanate-derived product from decomposition of the transiently inhibited complex, e-t; e-i, e-i′, and e-i″, irreversibly inactivated enzyme species.

cubation of the enzyme with a 300-fold molar excess of clavulanic acid for 2 h. Prolonged dialysis at room temperature against neutral buffer did not result in any significant reappearance of catalytic activity. This protein also shows an enhancement in its absorbance at 281 nm of about 1.3-fold (see Figure 1 (bottom)). The new chromophores in e-t and in e-i are very similar, and their intensities are proportional to the amount of inhibited (or inactivated) enzyme. While the chemical identities of these species cannot yet be defined, there are not many chromophores having a molar extinction coefficient of 20 000 and a λ_{max} of 280 nm that could reasonably come from an interaction between the enzyme and clavulanic acid. One possibility is a γ-amino, βγ-unsaturated carbonyl function (Ostercamp, 1970) that could derive from a clavulanic acid residue by elimination across the 5–6 bond as shown in Scheme II.

Reaction with Hydroxylamine. In order to see whether the inactivated protein is an acyl-enzyme, it was treated with hydroxylamine under conditions that are known to attack the ester groups of acyl-enzymes (e.g., Epand & Wilson, 1963; Inward & Jencks, 1965). Under these conditions (in which the activity of *native* enzyme is completely unaffected), 36% of the activity of irreversibly inactivated enzyme was regenerated (Figure 2). Concomitantly, the absorbance at 281 nm decreased essentially to the level of native enzyme (see Figure 1), both processes showing first-order dependence on hydroxylamine, with a rate constant of about 6 M^{-1} min^{-1} (30 °C, pH 7.0). This is similar to the rate constant observed by Holmquist et al. (1976) for the hydroxylamine-mediated deacylation of *O*-tyrosyl esters. [The rate constants for hydroxylaminolysis of esters of tyrosine, serine, and histidine are approximately 1.5, 0.001, and 2000 M^{-1} min^{-1}, respectively (Holmquist et al., 1976; Jencks & Carriuolo, 1959).] Even though the rate constant for the decrease in ultraviolet absorbance of the inactivated β-lactamase by hydroxylamine is unaffected by unfolding the protein in 1% sodium dodecyl sulfate and so is unperturbed by the enzyme's tertiary structure, the agreement with the literature value for an *O*-tyrosyl ester is only suggestive. The precise location(s) of the modified group(s) on the enzyme must await proper chemical characterization.

Three possibilities exist to explain the failure of hydroxylamine to restore the catalytic activity completely: (a) *all* the enzyme molecules become *partially* reactivated; (b) only one type of inactivated enzyme exists, but reaction with hydroxylamine produces 36% active enzyme and 64% inactive enzyme (this could happen if there was an anhydride link between inhibitor and enzyme, and if hydroxylamine attacked one carbonyl twice as fast as the other); and (c) multiple forms of inactivated enzyme exist, only one of which (comprising 36% of the total) can react with hydroxylamine. The first possibility seems unlikely in view of the fact that the fractional regain of catalytic activity is independent of the substrate used in the assay (i.e., benzylpenicillin, carbenicillin, phenoxymethylpenicillin, or cephalosporin C). Moreover, the K_m values for cephalosporin C, 6-aminopenicillanic acid, and 6-desaminopenicillanic acid (which range from 150 to 1200 μM) are the same when assayed with native enzyme or with the 36%-reactivated material under discussion. This makes it unlikely that the active site is partially blocked after hydroxylamine treatment. The second possibility was tested by using the radioactive nucleophile *O*-[^{14}C]methylhydroxylamine. This material restores a similar proportion of catalytic activity as does hydroxylamine, though it reacts some 30 times more slowly. Incubation of inactivated enzyme with *O*-[^{14}C]methylhydroxylamine, followed by gel filtration of the resulting mixture, showed that no radioactivity was associated with the partially reactivated enzyme. Ion-exchange chromatography

SCHEME II: Possible Pathways for the Generation of Inactivating and Chromophoric Species from Clavulanate.



of the small-molecule fraction from gel filtration showed a number of radioactive species, the pattern of which was similar to that obtained from an incubation of clavulanic acid itself with the radioactive nucleophile. This further suggests the possibility of an acyl-enzyme being responsible for the hydroxylamine-sensitive fraction of inactivated enzyme. The third possibility, that there is more than one type of inactivated enzyme, is established by the results described below.

Isoelectric Focusing of Inactivated Enzyme. When the inactivated enzyme is subjected to isoelectric focusing in polyacrylamide gel between pH 4 and 6, *three* species of inactivated enzyme appear in approximately equal amounts (see Figures 3 and 4), each of which has a lower p*I* than the native β-lactamase. On treatment with hydroxylamine, the material with the lowest p*I* disappears and returns to the position of native enzyme (Figure 4). Slab gel isoelectric focusing experiments were also performed in order to establish that similar results would be obtained if the inactivated enzyme was never exposed to either pH extreme. The results were identical with those obtained by the disc gel technique (in which such exposure is inevitable). There are, therefore, three species of inactivated enzyme, one of which is chromophoric and can react with hydroxylamine to regenerate the native enzyme (see Scheme I). The hydroxylamine-resistant inactivated enzyme (e-i′ and e-i″ in Scheme I) appears to contain no additional chromophore.

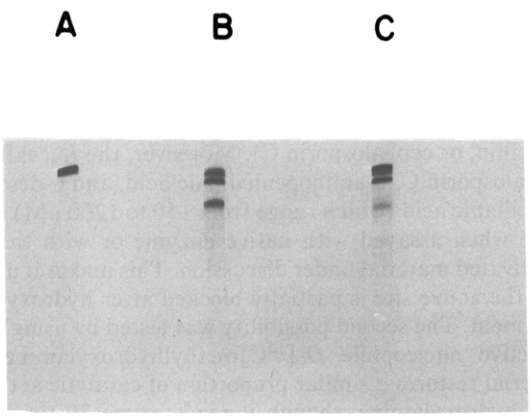


FIGURE 4: Polyacrylamide gel isoelectric focusing of inactivated β-lactamase. (A) Native enzyme; (B) enzyme inactivated by excess clavulanate; (C) sample B after partial reactivation by treatment with 20 mM hydroxylamine. The ΔpH range of these gels was approximately pH 4 to pH 6; the p*I* of native enzyme is 5.4.

Conclusions

In accordance with the findings described in this paper, the minimal mechanism for the interaction of clavulanic acid with the β-lactamase from *E. coli* RTEM must be expanded to that shown in Scheme I. Clavulanate depletion is catalyzed by the enzyme and involves loss of the β-lactam ring, a process that presumably involves clavulanate as a substrate. The transiently inhibited complex involves a clavulanate-derived chromophore that puts rather stringent limits on its structure. Analogous constraints apply to the fraction of the irreversibly inactivated enzyme that is reactivated by hydroxylamine, which also possesses a powerful chromophore. The changes in the ultraviolet absorbance, in the catalytic activity, and in the isoelectric focusing patterns when inactivated enzyme is treated with hydroxylamine, show the presence of three inactivated species only one of which can be reactivated. The multiplicity of products in this reaction means either that multiple sites of attachment exist on the enzyme each of which leads to inactivation, or that we are observing the consequences of the generation of a number of inactivating compounds by the action of the enzyme, or both. In any event, it seems that clavulanate is probably a "suicide reagent", and that the enzyme generates from it, one or more acylating agents (such as a ketene: cf. Bell et al., 1972; Woodward, 1977) or alkylating agents (such as a Michael acceptor). In Scheme II some possibilities are outlined that could in principle account for the chemical, spectroscopic, and isoelectric properties of the species e-t, e-i, e-i′, and e-i″ that are shown in Scheme I.

Although the interaction of clavulanic acid with the *E. coli* β-lactamase is not straightforward, we may hope to learn much about the enzyme and its inactivation by further scrutiny of this reaction.

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Inhibition of Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli* with Mono- and Bifunctional Arsenoxides†

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ABSTRACT: Pyruvate dehydrogenase multienzyme complex (PD complex) from *Escherichia coli* is comprised of three enzymes: pyruvate dehydrogenase (E1), lipoate acetyltransferase (E2), and lipoamide dehydrogenase (E3). Incubation of PD complex with pyruvate, coenzyme A, and *p*-aminophenyl arsenoxide ($H_2NPhAsO$; 100 μM) lead to a loss of 92% of the PD complex activity within 25 min at 4 °C. Controls lacking pyruvate and/or coenzyme A, but containing $H_2NPhAsO$, retained nearly all their PD complex activity. The loss of activity associated specifically with E3 was only 5% in the presence or absence of $H_2NPhAsO$. The arsenoxide formed a stable cyclic dithiolarsinite with reduced lipoic acid on E2 which was generated by pyruvate and coenzyme A according to the accepted reaction sequence within the complex. PD complex activity could be recovered to 78% within 2 min following the addition of 2,3-dithiopropanol (350 μM). Replacing $H_2NPhAsO$ with the bifunctional reagent $BrCH_2CONHPhAsO$ (100 μM) in the presence of pyruvate and coenzyme A caused a 100% loss in PD complex activity within 15 min. The loss of E3 activity was found to lag a few minutes behind the loss of PD complex activity and reached a value of 90% within 20 min. The initial reaction of the bifunctional reagent occurred on E2 via the R-AsO moiety and resulted in the rapid loss in PD complex activity. The inactivation of E3 was attributed to the subsequent delivery of the reagent into the active-site of E3. This is in keeping with the "swinging-arm hypothesis" ascribed to the flexible lysinyl–lipoyl group on E2 and suggests that the loss of E3 activity occurs via an active-site directed irreversible alkylation by the bromoacetyl moiety of the bifunctional reagent. At this stage E2 and E3 were cross-linked. Addition of 2,3-dithiopropanol failed to regenerate PD complex activity and E3 activity indicating that, although the reduced lipoic acid group on E2 was regenerated, the alkylation occurring in E3 was not reversed. Experiments with bromoacetylaniline ($BrCH_2CONHPh$;100 μM) caused only a slight loss of PD complex activity (10%) and E3 activity (3%) under conditions where the bifunctional reagent ($BrCH_2CONHPhAsO$) led to nearly complete inactivation of both activities. Monofunctional and bifunctional arsenoxides offer a new approach to the studies of 2-oxoacid dehydrogenase multienzyme complexes.

Pyruvate dehydrogenase multienzyme complex of *Escherichia coli* is comprised of three different enzymes that catalyze the overall reaction:

$$\text{pyruvate} + NAD^+ + \text{CoASH} \rightarrow \text{acetyl-CoA} + NADH + H^+ + CO_2$$

The enzymes, in order of their participation, are: pyruvate dehydrogenase (lipoate) (E1)[1] (EC 1.2.4.1), lipoate acetyltransferase (E2) (EC 2.3.1.12), and lipoamide dehydrogenase (NADH) (E3) (EC 1.6.4.3) (Reed, 1974; Hucho, 1975; Perham, 1975). E2 forms the core of the complex to which enzymes E1 and E3 bind in a noncovalent manner. The polypeptide chain stoichiometry of E1:E2:E3 approaches 2:1:1 (Bates et al., 1975, 1977; Perham & Hooper, 1977). A flexible "swinging-arm" of length 1.4 nm enables the reactive dithiolane ring of the covalently bound lipoic acid on E2 to interact with E1, coenzyme A, and E3 (Nawa et al., 1960; Koike et al., 1963; Green & Oda, 1961). Lipoic acid within the PD complex possesses a high degree of mobility (Ambrose & Perham, 1976; Grande et al., 1975). However, a single lipoic acid residue may not be able to span the distance of about 4.5 nm considered to exist between the active sites of E1 and E3 (Moe et al., 1974). Recent evidence (Danson & Perham, 1976; Brown & Perham,

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[1] Abbreviations used: PD complex, pyruvate dehydrogenase multienzyme complex; $H_2NPhAsO$, *p*-aminophenyl arsenoxide; $BrCH_2CONHPhAsO$, *p*-bromoacetylaminophenyl arsenoxide; $BrCH_2CONHPh$, bromoacetylaniline; E1, pyruvate dehydrogenase (lipoate) or pyruvate:lipoate oxidoreductase (decarboxylating and acceptor-acetylating) (EC 1.2.4.1); E2, lipoate acetyltransferase (EC 2.3.1.12); E3, lipoamide dehydrogenase (NADH) (EC 1.6.4.3).

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