Mechanism of Inactivation of Chymotrypsin by 5-Butyl-3*H*-1,3-oxazine-2,6-dione†

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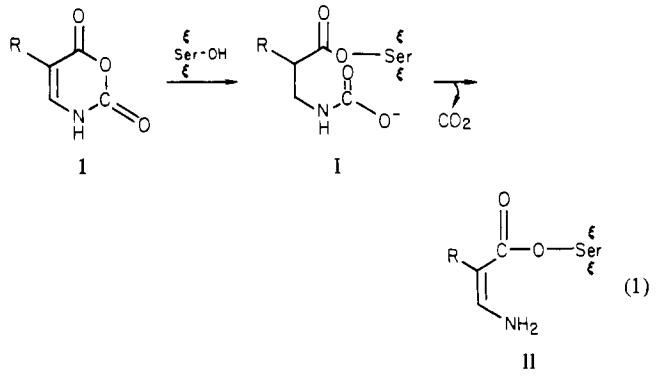
ABSTRACT: 5-Butyl-3*H*-1,3-oxazine-2,6-dione (**1**) inactivates chymotrypsin. The extent of inactivation is dependent upon the concentration of **1**. Upon dilution of the inactivated enzyme, catalytic activity is partially restored. Reactivation is a biphasic process. An initial relatively rapid phase (k = 1.8 × 10^{-2} min), whose amplitude is dependent upon the extent of dilution, is observed. Maximally, 60–65% of the catalytic activity can be recovered. The rapid phase is followed by a slow phase ($k \approx 1 \times 10^{-3}$ min^{-1}). With **1** labeled with ^{14}C at C-2, it was shown that two forms of inactive enzyme are formed, E·**1** and E·**1**′. ^{14}C label is retained in E·**1** but is no longer present in E·**1**′. Presumably, C-2 is lost as CO_2. The following reaction sequence is proposed for the inactivation of chymotrypsin: $E + 1 \rightleftharpoons E\cdot1 \xrightarrow{CO_2} E\cdot1' \rightarrow E + 1''$. The probable structures of E·**1**, E·**1**′, and **1**″ are shown in Scheme I in the text.

We have previously shown that oxazinediones inactivate chymotrypsin and other serine proteases (Moorman & Abeles, 1982). By analogy with the inactivation of chymotrypsin by isatoic anhydride, we had proposed that oxazinedione reacts with chymotrypsin to form an acyl-enzyme, which hydrolyzes slowly and, consequently, inactivates the enzyme. The mechanism of acyl-enzyme (II) formation is shown in eq 1.



(1)

Ser-OH = serine at the active site of chymotrypsin

Reaction with the OH of the active site serine results in ring opening and the formation of carbamate (I). We had assumed that this carbamate is highly unstable and decarboxylates rapidly to II. Due to the electron-releasing property of the amino group, II hydrolyzes slowly. There is considerable evidence that an electron-releasing group conjugated with the carbonyl group of an acylchymotrypsin reduces its rate of hydrolysis (Caplow & Jencks, 1962). In this paper, we report the results of an investigation of the mechanism of inactivation by 5-butyl-3*H*-1,3-oxazine-2,6-dione (**1**). These results require some modification of the of the mechanism shown in eq 1.

Experimental Procedures

Materials

α-Chymotrypsin (type I-S) was obtained from Sigma Chemical Co. and used without further purification. For gel filtration, Sephadex G-25 (fine) from Pharmacia Fine Chemicals was used. Barium [^{14}C]carbonate was obtained from New England Nuclear.

Methods

Analytical Procedures. All spectrophotometric determinations were made with a Perkin-Elmer Lamda 3 UV–vis spectrometer thermostated at 25 °C. For determination of radioactivity, samples were dissolved in 10 mL of scintillation fluid (ACS, Amersham) and counted on a Beckman LS-100 C liquid scintillation system. The counting efficiency was determined by addition of a ^{14}C-labeled standard (New England Nuclear). ^1H NMR spectra were recorded on a Varian EM 390 spectrometer with Me_4Si as a standard. Chymotrypsin concentration was determined spectrophotometrically by using E_{280} = 5 × 10^4 M^{-1} cm^{-1} (Dixon & Neurath, 1957). Chymotrypsin was assayed by adding aliquots of the enzyme to a solution of BzTyrOEt (3.6 mg/mL) in 0.1 M potassium phosphate, pH 6.8, containing 5% (v/v) acetonitrile (Hummel, 1959).

Preparation of Inactivated Chymotrypsin. α-Chymotrypsin (final concentration ca. 7 μM) was incubated with **1** (final concentration 30 μM) in 1 mL of 0.1 M potassium buffer, pH 6.8 (standard conditions). In some experiments [2-^{14}C]-**1** was used. After 10 min, the mixture was cooled in an ice bath and then applied to a Sephadex G-25 column (1 × 25 cm) at 4 °C and eluted with the same buffer. The fractions containing protein (~6 mL) were combined. On the basis of the specific radioactivity of the protein, the enzyme at this point contained 0.95 mol of **1**/mol of chymotrypsin and had 5–7% of the initial specific activity.

*Recovery of [2-^{14}C]-**1** from Inactivated Chymotrypsin.* A solution of inactivated chymotrypsin (1.15 μM) was prepared as described above. Unlabeled **1** (final concentration 0.6 mM) dissolved in acetonitrile was added to the enzyme solution. Aliquots (1 mL) were periodically removed and extracted with ethyl acetate (2 mL). Before the organic layer was analyzed for the amount of ^{14}C, a stream of N_2 was bubbled through it in order to remove dissolved $^{14}CO_2$. Aliquots of the ethyl acetate solutions were also chromatographed on silica plates as described in the synthesis of [2-^{14}C]-**1**. In both chromatographic systems, the radiolabel coincides with **1**. Control experiments were carried out which established that the extraction of **1** is quantitative (~98%) and that protein-bound **1** is not extracted.

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Synthetic Procedures. The synthesis of the 5-substituted oxazinediones was performed by the route of Washburne & Park (1976).

5-Butyl-3H-1,3-oxazine-2,6-dione (**1**). Ethyl carbamate (1.07 g, 12 mmol) and methyl 2-formylhexanoate (1.9 g, 12 mmol) were dissolved in 50 mL of benzene. Phosphorus oxychloride (615 mg, 4 mmol) was added, and the mixture was stirred for 2 h at room temperature. The phosphoric acid that was formed separated from the organic layer and was removed. The organic layer was washed with saturated NaCl solution, dried ($MgSO_4$), and evaporated. The colorless oil (2.5 g, 91%) was used without further purification for the subsequent cyclization. Polyphosphoric acid (50 mL) was heated to 80 °C. The oil obtained above (2 g) was added and stirred for 2 h at 75–80 °C. The hot mixture was poured onto a mixture of ice and ethyl acetate and was vigorously shaken until the polyphosphoric acid dissolved. The organic layer was washed with brine, dried ($MgSO_4$), and evaporated. The solid residue was purified by several washings with ether/petroleum ether: yield 1.05 g (71%); mp 125–126 °C; ^1H NMR ($CDCl_3$) δ 0.75–1.05 (m, 3 H), 1.1–1.6 (m, 4 H), 2.3 (t, 2 H), 7.8 (br s, 1 H), 9.3 (br s, 1 H). Anal. Calcd for $C_8H_{11}NO_3$: C, 56.80; H, 6.62; N, 8.22. Found: C, 56.80; H, 6.55; N, 8.28.

Methyl 2-Formylhexanoate. Diisopropylamine (10 g, 99 mmol) was dissolved in 100 mL of tetrahydrofuran. Butyllithium (66 mL, 1.5 mL in hexane) was added at −80 °C under an atmosphere of nitrogen and allowed to react for 30 min. Methyl hexanoate was added dropwise, and the mixture was stirred for 1 h at −80 °C. Methyl formate (6 g, 100 mmol) was introduced, and the reaction was warmed to room temperature over night. The mixture was poured over ice (100 g) and pentane (200 mL). The organic layer was discarded and the water phase extracted with ethyl ether (100 mL). The aqueous phase was acidified with diluted sulfuric acid. The oil, which separated, was extracted with ether. The ether solution was dried ($MgSO_4$) and evaporated. The oily residue was distilled in vacuo: bp 60–61 °C (1 mm Hg); yield 3.5 g (29%); ^1H NMR ($CDCl_3$) δ 0.9 (m, 3 H), 1.2–1.5 (m, 4 H), 1.7–2.2 (m, 2 H), 3.25 (dd, J_1 = 2.5 Hz, J_2 = 7 Hz, 0.7 H), 3.8 (s, 3 H), 7.0 (d, J = 13 Hz, 0.3 H), 9.7 (d, J = 2.5 Hz, 0.7 H), 11.4 (d, J = 13 Hz, 0.3 H).

5-Butyl-3H-[2-^{14}C]oxazine-2,6-dione. The procedure was the same as for the unlabeled compound but [1-^{14}C]ethyl carbamate was used. Procedures for the preparation of [^{14}C]ethyl carbamate and [^{14}C]diethyl carbonate are given below.

^1H NMR of [2-^{14}C]-**1** was identical with that of the unlabeled compound. The radiochemical purity was established by two thin-layer systems (silica, ethyl acetate, R_f 0.43; silica, methylene chloride/ethanol, 9:1, R_f 0.62). In both systems, the radiolabel coincides exactly with **1** (visible by UV_{254} fluorescence quenching). The specific radioactivity was 2.5 mCi/mmol.

[^{14}C]*Diethyl Carbonate.* The synthesis was performed according to the procedure described by Porter et al. (1976) with $BaCO_3$ (sp act. 5 mCi/mmol). The only modification was the use of low-boiling petroleum ether (bp 20–40 °C) rather than cyclohexane in the extraction of the product. This enabled us to isolate the product by evaporating the solvent at low temperature (−20 °C) in vacuo.

[^{14}C]*Ethyl Carbamate.* [^{14}C]Diethyl carbonate (157 mg, 1.45 mmol) was mixed with 5 mL of concentrated ammonium hydroxide and stirred for 2 h in a closed flask. Excess ammonia was evaporated in vacuo, and the resulting aqueous solution was saturated with NaCl and extracted with 50 mL of ether. The organic layer was dried ($MgSO_4$) and evaporated, leaving 60 mg (46%) of the ethyl carbamate as a crystalline solid. This solid was used to prepare [1-^{14}C]-**1** as described above.

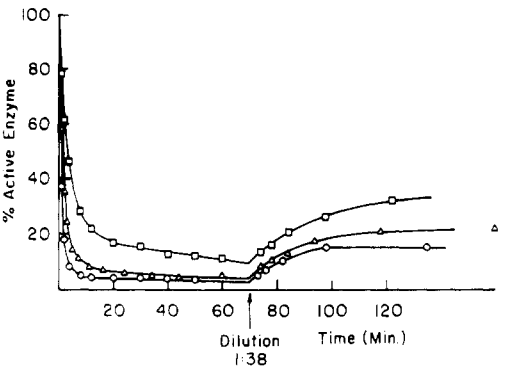


FIGURE 1: Inactivation of chymotrypsin by 5-butyl-3*H*-1,3-oxazine-2,6-dione (**1**). Chymotrypin (6.6 μM) was incubated with several concentrations of **1** under standard conditions. Aliquots were removed periodically and assayed for catalytic activity. After 70 min, the reaction mixture was diluted (1:38). Concentrations of **1**: 35 μM (○), 17.5 μM (△), 8.75 μM (□).

Results and Discussion

Reaction of 5-Butyl-3H-1,3-oxazine-2,6-dione (**1**) *with α-Chymotrypsin.* Chymotrypsin was allowed to react with **1** under the conditions of Figure 1. The reaction leads to a time-dependent loss of catalytic activity. Although the total amount of **1** present, in all experiments, exceeds the amount of enzyme, total inactivation does not occur. The fraction of enzyme inactivated depends on the concentration of **1**. Catalytic activity is restored upon dilution of the reaction mixture. The amount of catalytic activity restored depends on the extent of dilution. When the reaction is allowed to proceed without dilution (data not shown), a slow increase in catalytic activity occurs. The increase in catalytic activity suggests that the inactivator is converted to a compound that no longer inactivates chymotrypsin. The rate of reactivation is slow relative to the rate of inactivation and the return of activity upon dilution. The above data suggest that an equilibrium might be established between **1** and chymotrypsin. However, neither the fraction of enzyme inactivated at various concentrations of **1** nor the amount of activity recovered upon dilution corresponds to that which would be expected for a simple equilibrium. For instance, at 8.7 μM **1** 89% of the catalytic activity is lost. If that were an equilibrium process, $K_{eq} = 2.8 \times 10^6$ M^{-1}. The amount of activity that should be be recovered upon dilution of the three reaction mixtures (Figure 1) is 61, 49, and 31%, respectively. The data in Figure 1 show that this is not the case. The data suggest that reaction of **1** with chymotrypsin leads to the formation of two complexes, one of which can dissociate upon dilution to yield active enzyme and possible **1** and the other which cannot dissociate or dissociates slowly.

To further characterize the complex(es) formed between **1** and chymotrypsin, we synthesized **1** labeled with ^{14}C at C-2 and inactivated chymotrypsin with the labeled compound. The inactivation was allowed to proceed for 10 min, excess inactivator was then removed by gel filtration at 4 °C. The details of the experimental conditions are given in Figure 2. The enzyme at this point had 7% of the initial specific activity and contained 0.95 mol of **1**/mol of chymotrypsin. Clearly, loss of the C-2 carbon as CO_2 is not required for inactivation. The inactivated enzyme was then brought to 25 °C. Aliquots were removed periodically, and the loss of ^{14}C from the enzyme,

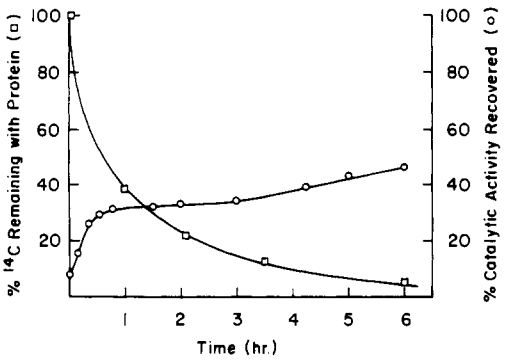


FIGURE 2: Loss of ^{14}C from chymotrypsin inactivated with 5-butyl-3*H*-1,3-[2-^{14}C]oxazine-2,6-dione (**1**). Chymotrypsin, 1.15 μM, inactivated with [2-^{14}C]-**1** was incubated at 25 °C under standard conditions. Aliquots were removed periodically and passed through a Sephadex G-25 column (1 × 25 cm) at 4 °C. The amount of ^{14}C-labeled material associated with the protein was monitored. An analogous reaction mixture containing chymotrypsin inactivated with nonisotopic **1** was periodically assayed for catalytic activity. (□) Percent of ^{14}C remaining in protein; (○) percent of original specific activity.

as well as the recovery of catalytic activity, was determined. The results are shown in Figure 2. Recovery of activity consists of a rapid phase followed by a slow phase. The amplitude of the rapid phase depends upon the concentration of the inactivated enzyme and increases with increasing dilution to a maximum recovery of approximately 60–65% of the original enzyme activity. When, under the conditions of Figure 2, the initial fast reactivation is complete (~45 min), approximately 30% of the catalytic activity is recovered, and 45% of the ^{14}C originally present in the enzyme remains associated with the enzyme. After 5 h, less than 10% of the original radioactivity remains with the enzyme, while only 45% of the catalytic activity is recovered. The data show that loss of ^{14}C from the enzyme is more rapid than recovery of catalytic activity. Therfore, an enzyme complex with a derivative of **1** must have been formed that no longer contains the C-2 carbon of **1** and that is not catalytically active. It is very likely that the C-2 carbon was lost as CO_2. Equation 2 shows a mechanism

$$\begin{array}{l} \mathrm{E} + \mathbf{1} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E}\cdot\mathbf{1} \xrightarrow[k_2]{\mathrm{CO_2}} \mathrm{E}\cdot\mathbf{1}' \xrightarrow{k_3} \mathrm{E} + \mathbf{1}'' \\ \qquad\qquad \downarrow k_2' \\ \qquad\qquad \mathrm{E} + \mathbf{1}'' + \mathrm{CO_2} \end{array} \qquad (2)$$

consistent with the data. According to that mechanism, **1** reacts with the enzyme to form a complex E·**1**. E·**1** can partition in three ways: it can dissociate to regenerate active enzyme (E) and intact inactivator, it can decompose to E and a derivative of **1** that no longer contains ^{14}C-2, or it can decarboxylate to give E·**1**′, a catalytically inactive species, which no longer contains ^{14}C. E·**1**′ can decompose, presumably by hydrolysis, to regenerate active enzyme (E). As stated above, dilution of the enzyme–inhibitor complex results, maximally, in recovery of 60–65% of the catalytic activity. This amount of activity recovered reflects partitioning of E·**1** between E and E·**1**′. Therefore, $(k_{-1} + k_2')/k_2 \approx 1.5$–1.9.

An experiment was carried out to verify that the reaction between chymotrypsin and **1** is reversible as required by the mechanism of eq 2. Chymotrypsin inactivated with [2-^{14}C]-**1** was prepared. The concentration of the enzyme was 1.15 μM, and it contained 0.95 mol of **1**/mol of chymotrypsin. To this solution was added nonisotopic **1** (0.6 mM, 500-fold excess). Periodically, aliquots were removed and analyzed for [2-^{14}C]-**1**. The results are shown in Figure 3A. A time-dependent release

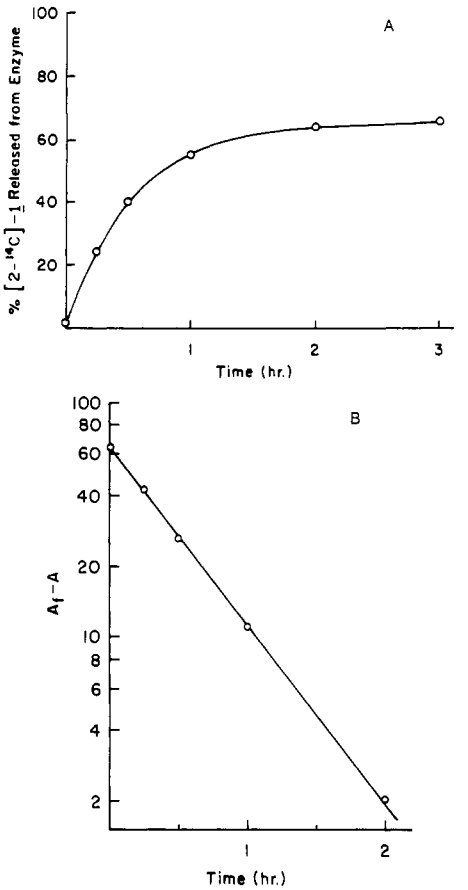


FIGURE 3: Recovery of 5-butyl-3*H*-1,3-oxazine-2,6-dione from inactivated chymotrypsin. (A) To a 1.15 μM solution of chymotrypsin inactivated with [2-^{14}C]-**1** was added 0.6 mM unlabeled **1**. Aliquots were periodically removed and assayed for nonenzyme-bound [2-^{14}C]-**1**. (B) The data in (A) are plotted on a semilogarithmic scale. A is percent of total ^{14}C released. A_f is percent of total ^{14}C released at t_∞ (65%). The rate constants k_{-1} and k_2 were calculated from the equations $k_{-1}/k_2 = A_f/(100 - A_f)$ and $k_{-1} + k_2 = 0.69/t_{1/2}$.

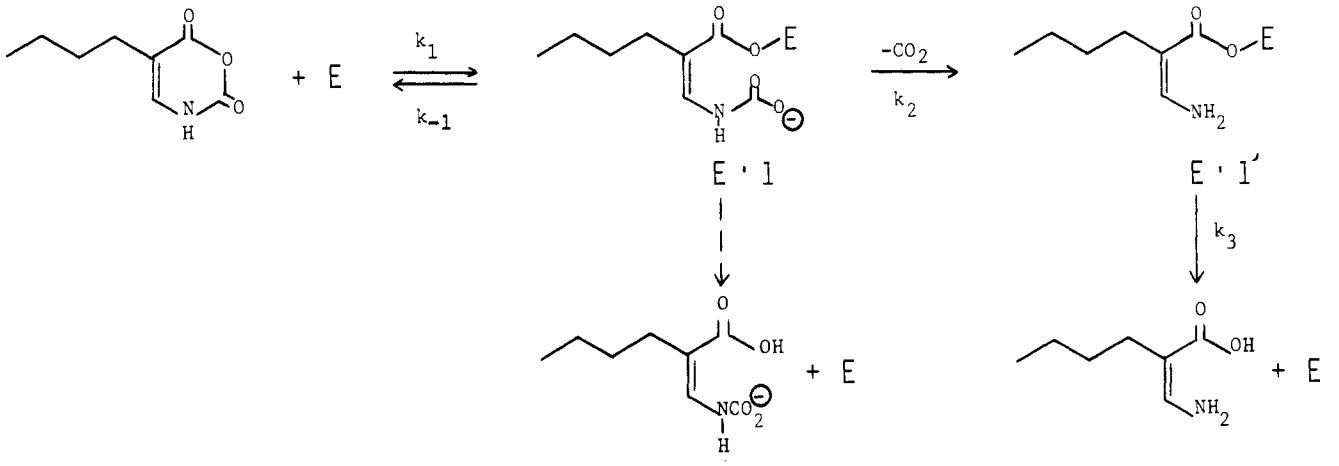
of **1** occurred. Maximally, 65% of radioactive material originally associated with the enzyme was released. The release of radioactive material is a first-order process (Figure 3B). After 2 h, essentially no radioactive material was associated with the enzyme. The data establish that the reaction of **1** with chymotrypsin is reversible as required by the mechanism of eq 2. Since only 65% of the radioactive material originally associated with the enzyme was recovered as **1** and there was no other radioactive material in solution, enzyme-bound **1** must have been converted to another intermediate (E·**1**′), which no longer contained the radioactive carbon. According to the mechanism of eq 2, E·**1** can partition to E + **1** (k_{-1}), E·**1**′ (k_2), and E + **1**″ (k_2').

E·**1**′ and **1**″ are derivatives of **1** that no longer contain C-2; i.e., they have lost the labeled carbon. Since only 65% of [^{14}C]-**1** was recovered, $k_{-1}/(k_2' + k_2) = 65/35 = 1.9$. The ratio $(k_{-1} + k_2')/k_2$ was evaluated above and found to be 1.5–1.9. Since the two values are very close, it follows that $k_2' < k_2$; i.e., $k_2' < 0.1k_2$. Therfore, decomposition of E·**1** through the pathway governed by k_2' is not important.

E·**1** breaks down by two pathways described by k_{-1} and k_2. The dissappearance of E·**1** should be a first-order process with $k_{obsd} = k_{-1} + k_2$. Figure 3B shows that E-**1** does decay in a first-order process with $k_{-1} + k_2 = 0.027$ min^{-1}. Since $k_{-1}/k_2 = 1.9$, $k_{-1} = 1.8 \times 10^{-2}$ min^{-1}, and $k_2 = 1 \times 10^{-2}$ min^{-1}.

Chymotrypsin and a stoichiometric excess of **1** were allowed to react under conditions of Figure 4. Aliquots were removed periodically, and the amount of protein-bound radioactive

Scheme I



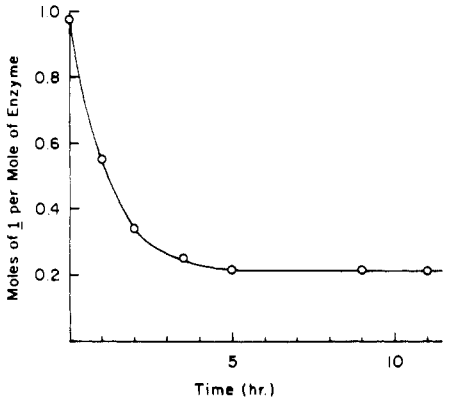


FIGURE 4: Reaction of chymotrypsin with 5-butyl-3*H*-1,3-[2-^{14}C]-oxazine-2,6-dione. The reaction mixture contained chymotrypsin (7 μM) and [2-^{14}C]-**1** (30 μM). Aliquots were removed periodically and passed through a Sephadex G-25 column (1 × 25 cm) at 4 °C. The amount of radioactive material associated with the protein was determined.

material was determined. The results are shown in Figure 4. Initially, a stoichiometric amount of **1** is bound to the enzyme. A gradual decrease of enzyme-bound **1** occurs until a steady state is reached. At steady state, the amount of radioactive material bound to the enzyme corresponded to 20% of the enzyme present; i.e., 20% of the enzyme is in the form of E·**1**. Under these conditions, 5% of the catalytic activity remains; therefore, 75% of the enzyme is in the form of E·**1**′. At steady state, k_2/k_3 = E·**1**′/E·**1** = 75/20. Since $k_2 = 1 \times 10^{-2}$ min, $k_3 \simeq 2.7 \times 10^{-3}$ min^{-1}. From the slow rate of recovery of catalytic activity (Figure 2), one can estimate $k_3 \approx 1 \times 10^{-3}$ min^{-1}.

The structure of E·**1** needs to be considered further. E·**1** could be a noncovalent complex between chymotrypsin and **1**, or it could be a covalent complex resulting from the addition of the OH group of the active site serine to the carbonyl group of the inactivator (as shown in Scheme I). To distinguish between the two possibilities, the adduct of [^{14}C]-**1** with chymotrypsin (1.15 μM) was prepared in a total volume of 4 mL and rapidly mixed with 0.5 mL of 1 N HCl. After addition of unlabeled **1**, the solution was extracted with 5 mL of ethyl acetate, to remove **1** from the aqueous phase. The extract contained less than 2% of the radioactive material present in the complex. Less than 1% of the radioactivity originally present was found in the aqueous layer. Therefore, upon denaturation of the enzyme, no intact **1** was released. Under these conditions **1** is stable. E·**1**, therefore, is not a noncovalent complex of **1** with chymotrypsin and is a covalent adduct. The probable structure of E·**1** is shown in Scheme I. When E·**1** is denatured, C-2 will be lost as CO_2.

The results reported here are consistent with the mechanism shown in Scheme I. According to the mechanism, –OH of the active site serine attacks the carbonyl group of **1** to form E·**1**, which can either dissociate to active enzyme and intact inactivator or decarboxylate to E·**1**′. In Scheme I, E·**1**′ is represented as an enamine, rather than an aldehyde. Although we have no direct evidence for the enamine, we prefer that structure. We believe that the electron-releasing properties of the enamine could be responsible for the slow hydrolysis of E·**1**′. Direct hydrolysis of E·**1** is slow relative to decarboxylation. The acyl-enzyme, E·**1**′, decomposes, probably by hydrolysis, to regenerate active enzyme and a product derived from **1**. The product released upon hydrolysis has not been identified. It should also be pointed out that we have no direct evidence that the active site serine is involved. Its proposed involvement is based on the normal catalytic reaction. The stability of E·**1**, a carbamate, is surprising ($t_{1/2}$ for decarboxylation is 29 min). Possibly, the stability is due to the unavailability of the proton required for decarboxylation.

Registry No. **1**, 89509-90-0; methyl 2-formylhexanoate, 89509-91-1; ethyl carbamate, 51-79-6; diisopropylamine, 108-18-9; methyl hexanoate, 106-70-7; methyl formate, 107-31-3; [^{14}C]ethyl carbamate, 73506-73-7; [^{14}C]diethyl carbonate, 62078-51-7; chymotrypsin, 9004-07-3.

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