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Interactions of Five- and Six-Membered Cyclic Esters with α-Chymotrypsin: Kinetic Evidence for Covalent Enzyme Intermediates

ELZBIETA IZBICKA[1] AND D. W. BOLEN

Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, Illinois 62901

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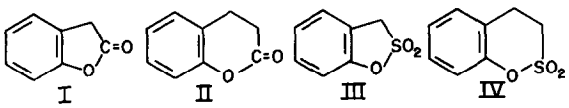
Interactions of α-chymotrypsin with 2-coumaranone (I), 3,4-dihydrocoumarin (II), *o*-hydroxy-α-toluenesulfonic acid sultone (III), and β-*o*-hydroxyphenylethanesulfonic acid sultone (IV) were studied in the presence of 14% acetonitrile at pH 7.0 by means of the proflavin displacement technique and by inhibition of *N*-acetyl-L-tryptophan ethyl ester (ATrEE) hydrolysis. Under saturating conditions of either I, II, or III, an enzyme intermediate was shown to accumulate using either the proflavin displacement technique or the ATrEE activity assay. The intermediates have characteristics of covalent enzyme–substrate compounds and are believed to decompose simultaneously by two pathways, one to give free enzyme and hydrolyzed cyclic ester, and the other to give the original cyclic ester and free enzyme. With α-chymotrypsin and III the observed first-order rate constant for decomposition of the intermediate by the two pathways was 0.19 ± 0.04 min^{-1}, while the rate constant for the hydrolytic pathway alone was 0.013 ± 0.0009 min^{-1}. These results indicate that the covalent-like intermediate with this sultone is not only capable of reverting to starting cyclic ester but prefers this pathway over hydrolysis. Sultone IV was found to bind to enzyme; but in contrast to the behavior of esters I–III, the binding did not result in accumulation of a covalent-like intermediate.

The finding by Kaiser and co-workers that certain five- and six-membered sulfonate, phosphate, and carboxylate cyclic esters are hydrolyzed by α-chymotrypsin has added an interesting variation to the mechanism by which the enzyme is believed to act (*1–3*). Many of their studies involving α-chymotrypsin made use of the 5-nitro derivatives of I and III as well as the 6-nitro derivatives of II and IV as substrates. These studies indicated that the nitro



I II III IV

cyclic ester derivatives formed covalent intermediates with the enzyme presumably by transesterification of cyclic ester to the serine 195 hydroxyl of α-chymotrypsin (*4*). Moreover, these authors suggested that the covalent inter-

[1] Current address: Zaklad Biochemii I-4, Polytechnika Wroclawska, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland.

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$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P$$

SCHEME A

mediates could readily revert (by k_{-2}) to their respective starting cyclic ester and enzyme as described by Scheme A. The reversibility of step 2 clearly distinguishes the enzymatic interactions of these cyclic esters from those of acyclic esters, which are essentially irreversible at step 2 under steady-state conditions.

A second aspect of this system concerns the ring strain which exists in some of the cyclic esters. Kinetic data on base-catalyzed hydrolysis of parent (**I–IV**) and nitro derivatives of cyclic esters suggested little ring-strain difference between **I** and **II** but significant apparent ring strain in **III** relative to **IV** (*5, 7*). Crystallographic studies confirmed the existence of ring strain in **III** compared to **IV**, the latter of which is considered to have essentially no ring strain (*6, 7*). Also, we recently have measured enthalpy changes for base hydrolysis of **I–IV** to provide quantitative indications as to the degree of strain in these compounds (*5*). Our results show that while **I** and **II** do not significantly differ in their heats of hydrolysis, sultone **III** is observed to be 98 kJ/mol more exothermic than **IV**. We have been unable to attribute this enthalpy change to any effect other than substantial ring-strain enthalpy associated with sultone **III**.

In the assumption that cyclic esters **I–IV** interact with α-chymotrypsin according to Scheme A, the question arises as to how the enzyme manages the energetics of ring opening on transesterification to the enzyme and reversion (by ring closure) to cyclic ester and free enzyme. In particular, one might predict that on transesterification of **III** with the enzyme, substantial ring-strain energy would be lost, making it very difficult to revert to sultone **III**. The significance of the energetics question rests entirely on the validity of the proposed mechanism—that is, whether transesterification does take place with the enzyme and equally important, whether such an enzyme substrate compound reverts significantly to starting ester. The work described here is designed to test the adequacy of Scheme A as a proposed mechanism for enzyme–cyclic ester interaction with particular emphasis on sultones **III** and **IV**.

Cyclic esters (**I–IV**) were used in these studies in preference to the nitro derivatives for two major reasons. First, the parent cyclic esters are considerably less subject to spontaneous hydrolysis in aqueous solution, a problem which can complicate interpretation of enzyme cyclic ester interactions. Secondly, if the cyclic esters react to form reversible covalent linkages with the enzyme as in Scheme A, it may be possible to measure enthalpy changes associated with the intermediates by direct calorimetric means. For this purpose the parent cyclic esters are preferred over the nitro derivatives since the nonnitrated compounds do not react as rapidly with the enzyme. Such conditions would make it easier to conduct a complete enthalpy analysis of the enzyme-mediated reaction.

MATERIALS AND METHODS

α-Chymotrypsin was purchased from Sigma Chemical Company and further purified on Sephadex G-75 by elution with 1 m*M* HCl (*8*). Active site titration of the purified preparation was 85–88% as assayed by *p*-nitrophenyl trimethylacetate (*9*) and this percentage did not change on lyophilization of the enzyme. Protein concentration was estimated assuming a molar absorptivity (280 nm) of 50,000 for α-chymotrypsin. All pH measurements were performed using a Beckman Model 3500 pH meter and the apparent pH recorded without correction for presence of acetonitrile.

α-Coumaranone (I) was obtained from Aldrich and recrystallized from CCl_4, mp 49°C, lit. 49°C (*10*). 3,4-Dihydrocoumarin (II) was purchased from Aldrich and redistilled under vacuum while *o*-hydroxy-α-toluenesulfonic acid sultone (III) (Eastman Kodak) was used without further purification (mp 87°C, lit. 86.1–87.1°C) (*11*). *o*-Hydroxyphenylethanesulfonic acid sultone (IV) was synthesized as previously described (*5*) and phenylmethane sulfonyl fluoride (PMSF) and *N*-acetyl-L-tryptophan ethyl ester (ATrEE) were obtained from Sigma.[2] Proflavin (3,6-diaminoacridine hemisulfate) was purchased from Calbiochem, recrystallized from water–ethanol and stored in the dark. Acetonitrile (Fisher, spectroquality grade) was redistilled over P_2O_5 in a nitrogen atmosphere. Water used in the experiments was deionized and millipore filtered (Super Q).

Proflavin Displacement—Type I Kinetics

In the experiments involving use of proflavin, the dye concentration was not higher than 120 μ*M* since deviations from Beer's law occur at higher concentrations (*12*). Concentration of proflavin was measured spectrophotometrically at 444 nm using a molar absorptivity of $3.34 \times 10^4\ M^{-1}\ cm^{-1}$ (*13*).

To observe the displacement of proflavin by a substrate, 2.7 ml of proflavin solution (20 μ*M*) in 0.03 *M* citrate buffer, pH 7.0, was placed in both the sample and reference cells and the spectrophotometer (Beckman Acta MVI) was set to zero absorbance at 465 nm. To the reference cell was added 0.2 ml of 0.03 *M* sodium citrate buffer, pH 7.0, followed by 0.1 ml of a 3 m*M* cyclic ester stock solution in CH_3CN. Then, 0.2 ml of the enzyme stock (1.5 m*M*) in 0.03 *M* sodium citrate buffer, pH 7.0, was added to the sample cell. The reaction was started by addition of 0.1 ml of the cyclic ester stock solution (3 m*M*) to the sample cell and the 465-nm absorbance change was observed over several minutes.

Proflavin Displacement—Type II Kinetics

The standard incubation mixture consisted of 0.05 ml of 1.2 to 1.6 m*M* α-chymotrypsin stock solution (freshly prepared from lyophilized enzyme) in 0.03 *M* citrate buffer, pH 7.0, mixed with 0.25 ml of 0.03 *M* citrate, pH 7, and

[2] Abbreviations used: PMSF (phenylmethane sulfonyl fluoride); ATrEE (*N*-acetyl-L-tryptophan ethyl ester), α-Ct (α-chymotrypsin).

0.05 ml of 12 to 15 m*M* cyclic ester stock dissolved in dry acetonitrile. Aliquots, 0.05 ml, were withdrawn from the incubation mixture at 3- to 5-min intervals and assayed for the amount of enzyme not covalently linked to cyclic ester.

The assays were of two types. One consisted of diluting an aliquot into ATrEE solution (2 m*M* in 0.03 *M* citrate, pH 7.0) and determining the initial velocity of ATrEE hydrolysis while the second assay consisted of diluting an aliquot into a proflavin solution and then measuring the amount of enzyme-proflavin complex formed at 465 nm.

The proflavin assay required the use of a double-beam spectrophotometer (Beckman Acta MVI). The absorbance was set at midscale with 3.0 ml of 120 μM proflavin in both the sample and reference cells and an aliquot (0.05 ml) of either a control incubation mixture or an incubation mixture containing cyclic ester and α-chymotrypsin was then added to the sample cell and the 465-nm absorbance was recorded.

Control incubation mixtures consisted of three types. One contained all components of the standard incubation mixture except 0.05 ml of CH_3CN replaced the 0.05 ml of cyclic ester in CH_3CN. A second control contained all necessary components except that the 0.05 ml of α-chymotrypsin solution was replaced with 0.05 ml of 0.03 *M* citrate buffer, pH 7.0. The third control incubation mixture consisted of all components, except 0.05 ml of 4 m*M* PMSF in CH_3CN was used in place of 0.05 ml of cyclic ester in CH_3CN.

The stability of the cyclic ester–enzyme intermediate was determined as a function of pH in the following manner. Aliquots of a standard incubation mixture at pH 7.0 were diluted into proflavin solutions which were maintained at pH 7.0, 5.8, and 4.21. The time course of the 465-nm absorbance increase was determined after dilution and the rate constants evaluated from first order plots. At pH values below 5.8 the decomposition of cyclic ester–enzyme was too slow to be measured.

pH-Stat Measurements

Measurements were performed on a Methrom pH-stat assembly consisting of Combititrator 3D, pH-meter E 300, Impulsomat E 373, and Dosigraph E 364-1.

Millipore-filtered water used for preparation of all solutions was boiled and degassed. Fresh solutions of NaOH were prepared daily from carbonate-free saturated stock solution. The apparent pH of NaOH solutions were in the range of 9.30 to 9.80 with each solution containing 0.1 *M* NaCl and 14% CH_3CN.

α-Chymotrypsin (20 mg) was dissolved in 1.9 ml of water and the pH of the solution was adjusted to 7.00 by slow addition of a maximum of 0.015 ml of 1 *M* NaOH with rapid stirring. When the pH was established, a 0.1-ml aliquot was withdrawn for determination of the enzyme concentration by absorbance at 280 nm. The reaction was initiated by addition of 250 μl of sultone **III** stock solution (59 m*M*) in CH_3CN or 250 μl of CH_3CN (control runs). The pH of the reaction mixture was maintained at 7.00 ± 0.03 by automatic addition of the base solution. Base addition due to autolysis was found to be negligible in comparison to enzyme-mediated sultone **III** hydrolysis and since the spontaneous hydrolysis rate

constant for III at pH 7 is about 2×10^{-4} min^{-1} (7), it was not considered to be a problem. The rate constant for α-Ct-mediated hydrolysis of III was determined from the maximum and constant rate of base addition established after about 18 min of reaction time (see results for further description). All pH-stat measurements were performed in a nitrogen atmosphere and maintained at 25.0°C with a Sodev programmable circulating thermostat.

RESULTS

Neither lactones I and II nor sultones III and IV possess spectral properties suitable for kinetic investigation of their interaction with α-chymotrypsin. Our inability to establish a convenient assay for α-chymotrypsin–cyclic ester interaction led us to use proflavin displacement as one means of observing such interactions. This technique has long been used to determine binding of inhibitors and substrates as well as to investigate the kinetics of substrate hydrolysis by α-chymotrypsin (*12–15*).

The method is based on the fact that proflavin, a competitive inhibitor of α-chymotrypsin, forms a 1 : 1 noncovalent complex with the enzyme which strongly absorbs at 465 nm (*16*). Addition of competitive inhibitor or substrate to an enzyme–proflavin mixture results in a decrease in 465-nm absorbance as the inhibitor binds to the enzyme active site and displaces the enzyme–proflavin equilibrium. Though weak substrate–proflavin complexes may form, their absorbance differences at 465 nm are quite small in comparison to the absorbance change due to proflavin displacement from the enzyme active site (*16*).

Basically two types of experiments (type I and type II) are described which permit elucidation of various aspects of the cyclic ester–enzyme interaction. In the type I experiment, proflavin is incubated with an excess of α-chymotrypsin, and sufficient cyclic ester is added to equal the concentration of enzyme. These conditions were chosen in order to limit enzyme turnover and test whether cyclic esters may behave according to the mechanism of Scheme A. The results of such experiments with lactones I and II and sultones III and IV are given in Fig. 1. The time course of proflavin displacement suggests that the α-chymotrypsin interaction with cyclic esters I, II, and III goes beyond simple binding, but that the behavior of sultone IV is consistent with simple binding. Though useful in illustrating that complex formation occurs, this (type I) experiment gives little information as to the nature of the complex(es) formed between enzyme and cyclic ester.

A more refined method which allows one to measure the extent of formation of what is believed to be a covalent complex is described by the type II kinetic experiments. In this series of experiments we entertain the possibility that the cyclic esters may follow the mechanism of Scheme A, and our purpose is to determine whether or not enzyme–ester intermediates accumulate during the course of reaction. The experiments were performed by incubating enzyme with excess cyclic ester and then removing aliquots from the incubation mixture for assay of the amount of kinetically competent enzyme (ATrEE assay) or enzyme

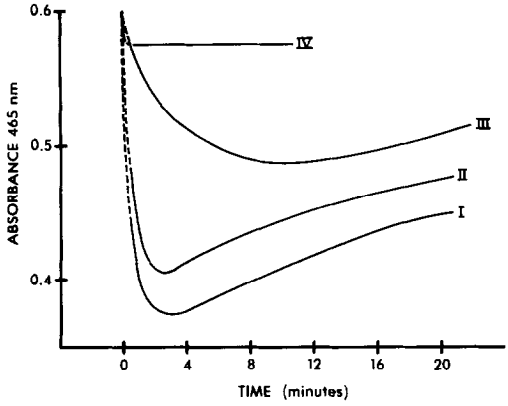


FIG. 1. Proflavin displacement (type I) as an assay for α-chymotrypsin–cyclic ester interaction. α-Chymotryspin was incubated with proflavin in a 2.9-ml total volume containing 0.03 *M* citrate buffer, pH 7.0, at 25.0°C. Proflavin displacement was initiated by addition of 0.1 ml stock solution of the indicated cyclic ester in CH_3CN. Final concentrations were 100 μM α-chymotrysin, 100 μM cyclic ester, 20 μM proflavin, and 14% (v/v) CH_3CN.

still capable of binding proflavin (proflavin displacement assay).

In the case of the proflavin displacement assay, timed aliquots of the incubation mixture were diluted into solutions of excess proflavin which scavenges essentially all enzyme not tied up as covalent enzyme–ester compound. The 465-nm absorbance gives a direct measure of $E_{total} - EA$, and Fig. 2 illustrates the results of type II experiments using the indicated lactones and sultones. For the experiment, both sample and reference cells of the spectrophotometer contained high concentrations of proflavin (120 μM), and the recorder pen was adjusted to midscale (indicated as zero absorbance units).

In order to establish the boundary conditions (ΔA_{max} and ΔA_{min}) for absorbance changes, several controls were then performed. The maximum 465-nm-absorbance change possible would be observed if no covalent enzyme compound was formed between cyclic ester and α-Ct. This was determined by use of a control incubation mixture containing exactly the same concentrations of components but without cyclic ester. The ΔA_{max} observed is indicated by the maximum positive change in absorbance in Figs. 2A and B. The minimum absorbance change possible (ΔA_{min}) would be observed if all of the enzyme in the incubation mixture became tied up as covalent enzyme–ester compound. Under such circumstances no proflavin binding would occur on addition of an aliquot of the incubation mixture to the proflavin assay. This possibility was simulated in two ways. In one, a control incubation mixture was prepared containing all components except that 0.03 *M* citrate buffer, pH 7.0, replaced α-chymotrypsin. The ΔA_{min} observed is indicated as the negative absorbance change in Figs. 2A and B. This control simply indicates dilution of the proflavin in the sample cell by the incubation mixture aliquot. The second type of control was performed by replacing the cyclic ester in the incubation mixture with phenylmethane sulfonyl fluoride (PMSF). This substrate rapidly forms an irreversible (covalent) compound with the

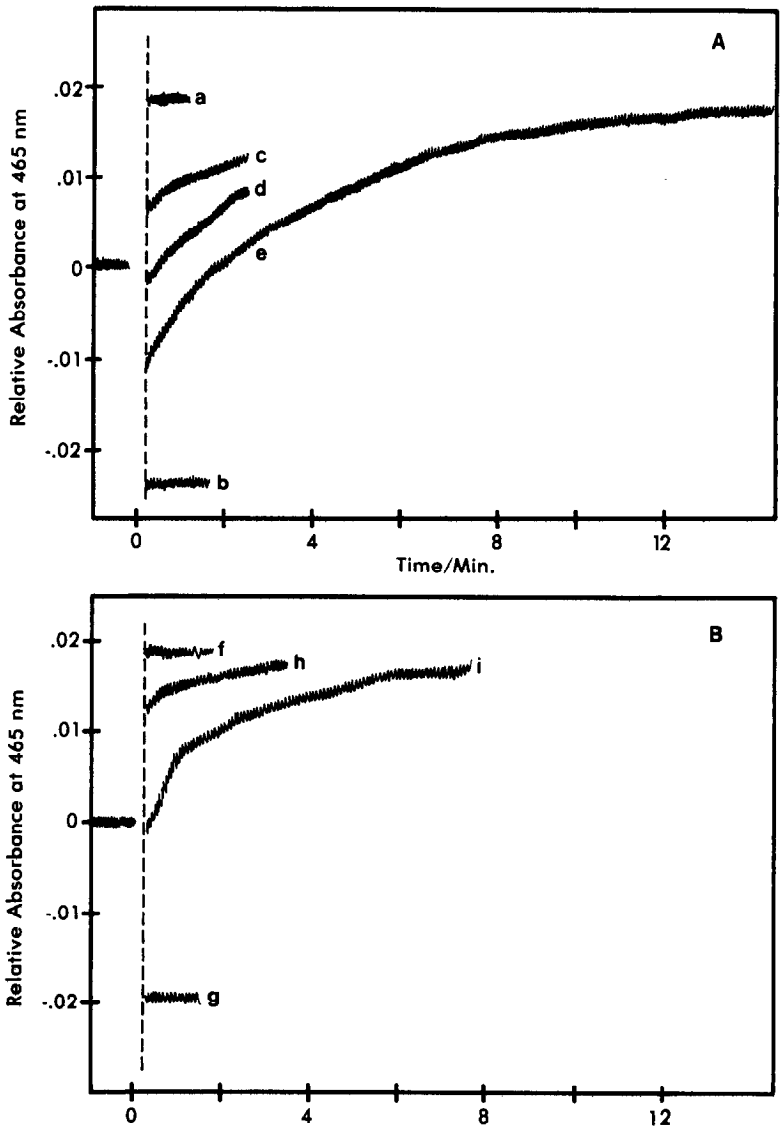


FIG. 2. Proflavin displacement (type II) as an assay for α-chymotrysin–cyclic ester interaction. The recorder was set to 50% of full scale (465 nm and 0.1 absorbance) with 3.0 ml of 120 μM proflavin in 0.03 M citrate buffer, pH 7.0, and 25.0°C in both sample and reference cells. The resulting conditions are indicated in A and B as zero absorbance. (A) (a) Absorbance observed on delivering an aliquot (50 μl) of a control incubation mixture (containing 200 μM α-Ct, 0.03 M citrate, pH 7.0, and 14% (v/v) CH_3CN) to the sample cell (ΔA_{max}). (b) Absorbance observed on delivering 50 μl of a control incubation mixture (containing 2 mM sultone III in 0.03 M citrate, pH 7.0, and 14% CH_3CN) to 3.0 ml proflavin solution in the sample cell (ΔA_{min}). (c) Absorbance change on delivering 50 μl of an incubation mixture to 3.0 ml of proflavin in the sample curvette, 2 min after initiating the incubation mixture. The incubation mixture consisted of 200 μM α-Ct, 2 mM sultone III, and 14% (v/v) CH_3CN in 0.03 M citrate buffer, pH 7.0, at 25°C. (d) Absorbance of a 50-μl aliquot from the same incubation mixture as in (c) but after 9 min incubation time. (e) Repeat of (d) but after 35 min incubation time. (B) Experimental procedure was the same as in A. (f) Absorbance control as in (a) above. (g) Absorbance control as in (b) above except 2 mM lactone I or II replaced sultone III. (h) Absorbance of a 50-μl aliquot of an incubation mixture containing 200 μM α-Ct, 2 mM lactone *II* in 0.03 M citrate, pH 7.0, and 14% CH_3CN. Incubation time was 10 min. (i) Same as in (h) except 2 mM lactone I was used and the incubation time was 4 min.

enzyme active site (*17*) and prevents proflavin from binding. An aliquot of this incubation mixture gave a ΔA_{min} value identical to that of the dilution control. The other absorbance changes indicated in Figs. 2A and B represent specific time points from cyclic ester–α-chymotrypsin incubation mixtures.

An alternative way of assaying for the amount of enzyme not tied up as covalent enzyme–cyclic ester intermediate consisted of diluting aliquots of the incubation mixture into solutions containing high concentrations of specific substrate (ATrEE). In this case the initial velocity for ATrEE hydrolysis directly reflects the amount of catalytically competent enzyme, a quantity which is observed to decrease with the time of cyclic ester–enzyme incubation (Fig. 3). This suggests that covalent enzyme–substrate compound is proceeding in the incubation mixture, and that a plot of the ATrEE initial velocities relative to the initial velocity at zero incubation time could give an indication of the rate of the accumulation of an intermediate. ATrEE hydrolysis rate data (Fig. 3) plotted in this manner are given in Fig. 4 for sultones **III** and **IV**. Also given in Fig. 4 are the relative (instantaneous) observed 465-nm-absorbance changes (see Fig. 2A) using the proflavin assay to monitor the amount of enzyme still capable of binding proflavin. Both the ATrEE and the proflavin assays indicate that sultones **III** and **IV** differ markedly in their manner of interaction with enzyme.

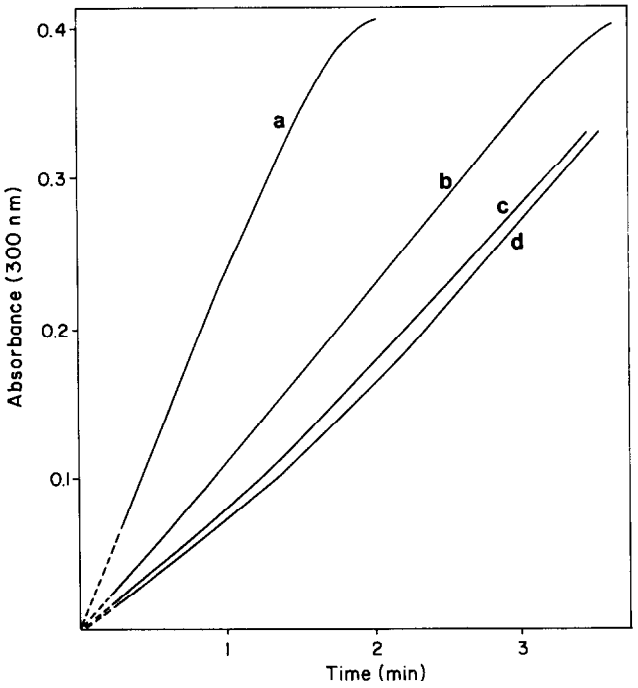


FIG. 3. Interaction of α-chymotrypsin with sultone III (type II) as monitored by enzymic activity assay with ATrEE. Incubation mixture containing α-Ct, sultone III, and CH_3CN was identical to that of Fig. 2A. Aliquots of 10 μl were taken from the incubation mixture at the indicated times and assayed for ATrEE activity. Incubation times: (a) $t = 0$ (fastest initial velocity); (b) 5 min; (c) 20 min; (d) 30 min (slowest initial velocity).

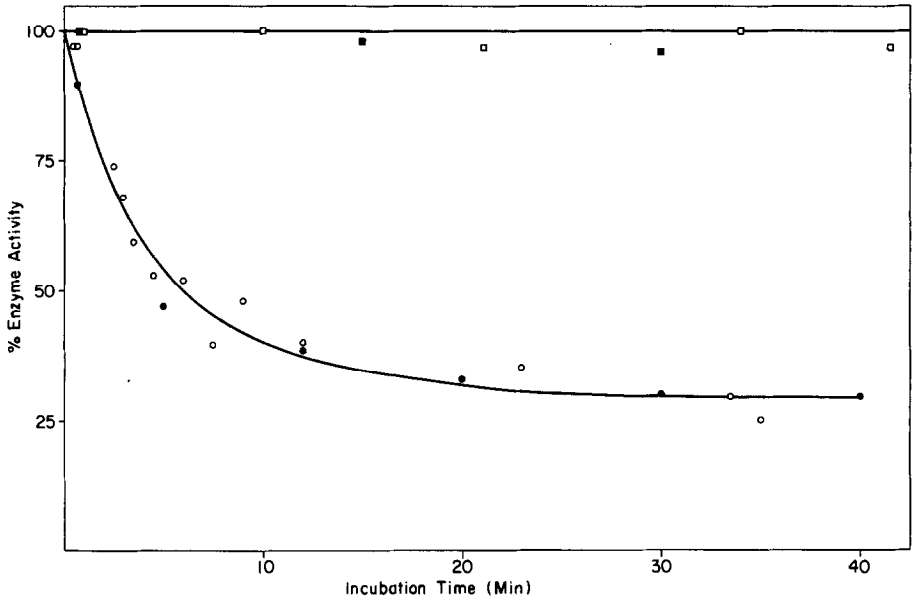


FIG. 4. Extents of rates of accumulation of intermediates from the interactions of III and IV with α-chymotrysin. Aliquots of incubation mixtures containing 14% CH_3CN, 200 μ*M* α-Ct, and 2 m*M* sultone III (○, ●) or IV (□, ■) in 0.03 *M* citrate buffer, pH 7.0, were assayed at timed intervals for the percentage active enzyme remaining. The instantaneous absorbance changes from proflavin displacement assays, e.g., Fig. 2A (open symbols), are plotted as a function of incubation time along with ATrEE activity measurements (filled symbols) as for example in Fig. 3.

Not only are the data in Figs. 2A and B useful in defining the rate of accumulation of a cyclic ester–enzyme intermediate (instantaneous absorbance change), but the further absorbance changes after dilution are also of value. Once the incubation aliquot is diluted into the proflavin solution the 465-nm absorbance is seen to increase to the ΔA_{max} value expected when all the enzyme is bound to proflavin. That is, all of the enzyme is eventually returned to the state in which it maximally binds to proflavin. The rate of return to free enzyme was found to be strictly first order, and an example of such data (35-min incubation time of Fig. 2A) is given in Fig. 5.

While the above experiments reflect the nature of intermediates on interaction of α-Ct with cyclic esters, no data are provided on the overall reaction. Such information can be obtained by observing the acid product of cyclic ester hydrolysis. In terms of Scheme A, hydrolysis of EA is accompanied by release of a hydrogen ion for each molecule of acid formed, and the hydrogen ion is detectable by potentiometric titration. For sultone III, pH-stat measurements were performed by adding a solution of III in acetonitrile to the unbuffered α-Ct solution maintained at pH 7.00 ± 0.03. Initially, about 50 μl of base was immediately added to maintain pH because of the perturbation caused by acetonitrile and interaction of III with α-Ct. After addition of this initial aliquot of base, the rate of base addition was quite slow but became progressively more

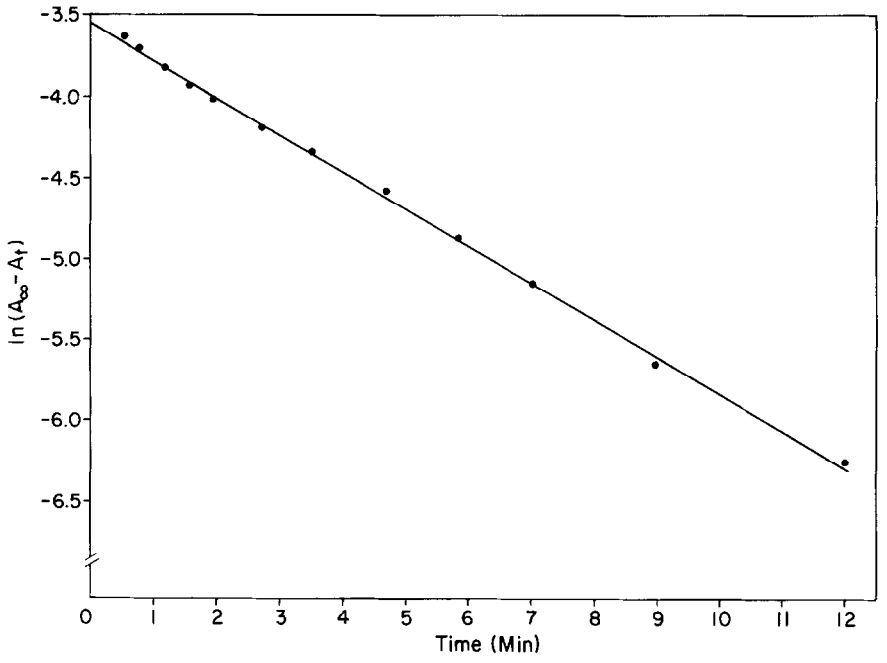


FIG. 5. Decomposition rate of the putative sulfonyl enzyme formed from α-Ct and sultone III. Data were taken from curve (e) of Fig. 2A and an observed rate constant of 0.22 min^{-1} was determined by least-squares fit.

rapid with time reaching a maximum constant rate of addition after 18 to 20 min. The behavior of the pH-stat measurements correlates well with the data of Fig. 4, which show that a maximum accumulation of intermediate (EA) also occurs after 15 to 20 min.

In reference to Scheme A, the constant maximum rate of formation of *o*-hydroxy-α-toluenesulfonic acid should be equal to k_3[EA]. By knowing that the steady-state amount of EA in the plateau region of Fig. 4 is around 70% of total enzyme, a k_3 of 0.013 ± 0.0009 min^{-1} was evaluated.

DISCUSSION

Several observations can be made concerning Fig. 1, which reveal the result of adding cyclic ester to an α-chymotrypsin–proflavin mixture. First, proflavin displacement is observed with all of the cyclic esters, indicating that they all bind to the enzyme active site.[3] Secondly, the extents of maximum proflavin displacement (465-nm absorbance decrease) differ among the cyclic esters, with lactones appearing to have significantly greater affinity for the active site than the sultones.

[3] Additional support is given by the finding that cyclic esters III and IV are competitive inhibitors of ATrEE hydrolysis under conditions in which there is essentially zero incubation time of sultone with α-Ct. (Izbicka and Bolen, unpublished results.)

Thirdly, under the conditions in which roughly one turnover of enzyme would occur with cyclic ester ($[E_{total}]$ = [cyclic ester]), lactones I and II and sultone III exhibit first a decrease in 465-nm absorbance followed by a slow increase. This behavior can readily be explained if these esters interact with α-chymotrypsin according to the mechanism of Scheme A. If we assume that the initial proflavin displacement signifies binding and formation of covalent ester intermediates, then subsequent hydrolysis of the covalent enzyme species would release enzyme which would be free to bind proflavin (increase in 465-nm absorbance). *N-trans*-Cinnamoylimidazole, which reacts with α-chymotrypsin to form a covalent (acyl–enzyme) intermediate, exhibits absorbance changes in the presence of proflavin similar to those found with cyclic esters I, II, and III (*14*). Thus, the behavior of the three cyclic esters is at least consistent with the mechanism described by Scheme A.

The final point of interest concerning Fig. 1 is the behavior of sultone IV. The apparent extent of binding is about 20% of the maximum observed for sultone III; and the data also differ in that after binding, no further changes appear to take place. This could either mean that only noncovalent binding occurs between IV and α-chymotrypsin or that if a covalent sulfonyl–enzyme is formed, it does not hydrolyze. As discussed later, we believe that the former possibility is correct and that sultone IV does not form a sulfonyl–enzyme intermediate detectable under our conditions.

If we take Scheme A as an adequate description of the behavior of cyclic esters I, II, and III in Fig. 1, then it must be concluded that formation of the EA intermediate and subsequent hydrolysis are both rather slow processes. Furthermore, k_2, k_{-2}, and k_3 are all likely to be small, which should make it possible to trap kinetically the putative covalent intermediate. On the basis of these considerations, experiments (type II) were designed to investigate the nature of complexes formed between cyclic ester and α-chymotrypsin. Such experiments consisted of incubating α-chymotrypsin with excess cyclic ester and then removing aliquots for assay of the amount of enzyme not tied up as a covalent complex. (In all cases the cyclic ester concentration was either saturating or, in the case of IV, near saturation.) The assay systems into which the aliquots were placed possessed high concentrations of either ATrEE or proflavin. Both assays function by quantitating the amount of enzyme which is not covalently linked to compounds I, II, III or IV.

ATrEE Assay

The situation which prevails on diluting an aliquot of cyclic ester–enzyme incubation mixture into an ATrEE assay system is that ATrEE $\gg$ cyclic ester $>$ α-Ct. The 60-fold dilution tends to shift rapidly the noncovalent α-Ct: cyclic ester form of the enzyme to free enzyme and cyclic ester, and the proportionately large excess of ATrEE sequesters essentially all of the enzyme not tied up as covalent cyclic ester–enzyme complex. Thus, the initial velocities for ATrEE hydrolysis should be directly proportional to the total enzyme present minus the covalently bound complex. A plot of the fractional inhibition obtained from α-Ct–III and α-Ct–IV incubation mixture aliquots as a function of incubation time (Fig. 4)

illustrates the very different behavior exhibited by the two sultones. Similar plots (not shown) for α-Ct–I and α-Ct–II incubation mixtures exhibit the same behavior as α-Ct–III but with more rapid and extensive accumulation of intermediate.

It is important to point out the unusual behavior of the hydrolysis of ATrEE on adding an aliquot of the cyclic ester α-Ct (Fig. 3 gives results with III) incubation mixture to the ATrEE assay system. The hydrolysis is slow at first and becomes more rapid with time. This sort of activation is exactly what would be expected if a covalent cyclic ester–enzyme complex begins to decompose during the course of the assay to generate free enzyme, capable of catalyzing ATrEE hydrolysis.

In principle, one should be able to estimate the EA decomposition rate constant ($k_{-2} + k_3$) (Scheme A) from the first derivatives along the curved lines of Fig. 3. In practice the constants would only be approximate, since the rate of ATrEE hydrolysis is dependent not only on the amount of competent enzyme but also on the prevailing concentration of ATrEE and competitive inhibition by sultone III and *N*-acetyl-L-tryptophan. If we restrict the evaluation of tangents to the region below 0.2 absorbance units, the inhibition and substrate depletion effects are diminished and ($k_{-2} + k_3$) can be evaluated using

$$(k_{-2} + k_3) = \frac{1}{t} \ln\left(\frac{V_0 - V_i}{V_0 - V_t}\right). \qquad [1]$$

Here, V_0 is the uninhibited velocity (no sultone present), V_i is the initial velocity of the sultone containing reaction mixture, and V_t is the tangent at time, t. The evaluated ($k_{-2} + k_3$) constants were highly variable, ranging from 0.08 to 0.16 min^{-1}, and were found to be quite dependent on V_0, which itself has a 5–10% error. These values are to be compared with the 0.19 ± 0.04 min^{-1} value for ($k_{-2} + k_3$) determined from proflavin displacement data (see below). Given the errors and approximations associated with the ATrEE data, we believe agreement between the two independent sets of data is favorable.

Proflavin Assay

Dilution of an aliquot of incubation mixture into excess proflavin results in formation of enzyme–proflavin complex with the available free enzyme (Figs. 2A and B). Analogous to the ATrEE assay, the 60-fold dilution and presence of excess proflavin results in proflavin binding of all enzyme not covalently linked to ester. Thus, the initial 465-nm absorbance on diluting an incubation mixture aliquot into the proflavin assay system is a measure of $E_{total} - EA$.

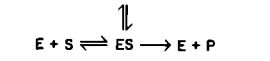
A plot of the fraction of enzyme not covalently linked to sultone III and IV as a result of the proflavin assay is given in Fig. 4 along with the results using the ATrEE assay. There is good agreement between both assays, indicating that they are responding to the same phenomenon. While sultone III appears to form a compound with α-chymotrypsin which can be detected by the ATrEE and proflavin assays, sultone IV gives no evidence of having formed a compound of sufficient magnitude and stability to be detected under the experimental conditions. It is very doubtful that a transient α-chymotrypsin-IV covalent complex would have formed in any case, given the fact that IV is an extremely unreactive

ester (*5–7*). In light of the results of type I and type II experiments using sultone **IV**, the only statement which can be made is that it binds to the active site of α-chymotrypsin, but only noncovalently.

The plateau level of 70–75% EA (Fig. 4) in the incubation mixture is a steady-state concentration which gives no information as to the rate of EA decomposition. This decomposition rate can readily be measured by diluting an aliquot of the incubation mixture into the proflavin assay system (Fig. 2A). The 60-fold dilution of cyclic ester along with the high concentration of proflavin tremendously reduces the ability of cyclic ester to bind to α-chymotrypsin in the assay mixture; and in terms of Scheme A, the decomposition should be first order with a rate constant of $(k_{-2} + k_3)$. We have found that decomposition is invariably first order (Fig. 5) and is a function of pH. In fact, the observed decomposition rate constant at pH 5.8 is approximately 15-fold less than at pH 7, and at pH 4.2 the decomposition is too slow to obtain good rate data. The decrease in the rate of EA decomposition at acid pH formed the basis for our attempts to isolate and characterize the α-chymotryspin-**III** compound, as described in the following paper.

The data presented here provide strong evidence for the reversible accumulation of cyclic ester–chymotrypsin complexes (with esters **I**, **II**, or **III**) which have properties expected of covalent intermediates. However, these observations do not indicate whether the accumulated (EA) complex lies on the path to product formation (Scheme A) or whether it is simply a dead-end complex as illustrated in Scheme B. Fortunately, it is possible to distinguish between mechanisms A and B by observations of the rate of formation of hydrolyzed product as a function of time after mixing excess cyclic ester (e.g., **III**) with enzyme. Scheme A would result in a rate of product formation proportional to the concentration of EA, thus achieving a maximum rate after 15 to 20 min (see Fig. 4). Scheme B, if operative, would result in the most rapid rate of product formation immediately on mixing cyclic ester with enzyme; and the rate should be inverseiy proportional to [EA], reaching a minimum rate after 15 to 20 min. The actual measurement (pH stat) of the rate of *o*-hydroxy-α-toluenesulfonic acid production mixing **III** with α-chymotrypsin begins with a slow rate of product formation which increases to a maximum after 18 min. This directly demonstrates that the accumulating complex lies on the path leading to sultone **III** hydrolysis, and the simplest mechanism which describes the data is Scheme A.

The rate constant, k_3, was evaluated from the maximum and constant rate of formation of *o*-hydroxy-α-toluenesulfonic acid (pH-stat measurement, with **III** saturating) along with knowledge of the total amount of enzyme tied up as EA (i.e., 70%). The value obtained ($k_3 = 0.013 \pm 0.0009$ min^{-1}) is more than an order of magnitude lower than $k_{-2} + k_3$ (0.19 ± 0.04 min^{-1}). This demonstrates conclusively that k_{-2} is at least a factor of 10 larger than k_3 and that reversion of EA to sultone **III** is preferred over hydrolysis.



SCHEME B

If EA of Scheme A is a complex formed by transesterification of sultone **III** to the enzyme, its preference for reversion to **III** is a curious phenomenon considering the highly strained nature of the five-membered sultone ring (*5–7*). One might have expected a considerable release of ring-strain energy on transesterification which would result in a much lower free energy of EA than suggested by the apparent equilibrium between ES and EA.

There are several plausible explanations which we are investigating to account for the phenomenon. Since we have very limited knowledge of the thermodynamics of sulfonate transesterification in an organic mileau, it may be that the process is not overly favorable despite the high degree of ring strain in **III**. It is also possible that the enzyme is simply not permitting the release of ring-strain energy, and the covalently bound substrate EA represents an energy-poised state. Finally, up to this point we have assumed that transesterification to serine 195 is the site of attachment;[4] however, histidine 57 (sulfonylimidazole formation) is also a candidate, and since this linkage would likely be of higher free energy than a serine ester linkage (*18*), this possibly could explain the phenomenon. We have attempted to resolve this latter question by isolation of the EA complex and by trying to distinguish between the His 57 and Ser 195 sites of attachment. The results of these efforts are described in the paper which follows.

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[4] Using the 5-nitro derivative of sultone **III** as a substrate, Kaiser and co-workers have shown that the nitrophenolate chromophore of the resulting sulfonyl enzyme exhibits a perturbed titration curve suggesting an ionized group in close proximity to the nitrophenolate chromophore. Additional pH-dependent sulfonylation and desulfonylation rate data suggest this ionized group is imidazole of histidine 57 and the most likely site of attachment of the sulfonyl moiety is serine 195 (*19*).

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