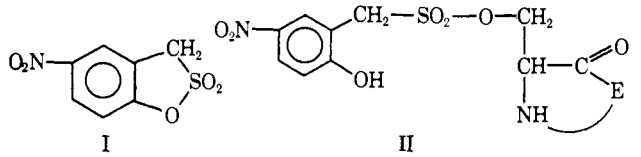
Studies on the Desulfonylation of 2-Hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin

J. H. Heidema[1] and E. T. Kaiser[2]

Contribution from the Department of Chemistry, University of Chicago, Chicago, Illinois 60637. *Received April 10, 1970*

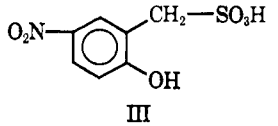
Abstract: Reaction of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone with serine-195 at the active site of α-chymotrypsin produces a sulfonyl enzyme which undergoes desulfonylation under conditions where α-toluenesulfonyl-α-chymotrypsin is stable. The rate of desulfonylation of 2-hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin (II) has been measured using a large excess of the specific substrate *N*-acetyl-L-tryptophan methyl ester which rapidly scavenges any free enzyme. One pathway for desulfonylation involves attack of the phenolic hydroxyl group in II on the sulfonyl function to regenerate the starting sultone. This reaction appears to involve the catalytic participation of the active-site residue histidine-57 and is among the first examples where a reactive function introduced into an enzyme molecule has been shown to act as an intramolecular nucleophile.

We have shown that 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I) reacts rapidly and stoichiometrically with the active site of the proteolytic enzyme α-chymotrypsin (CT) to produce a catalytically inactive sulfonyl enzyme II.[3] This sulfonyl enzyme then decomposes in a much slower first-order reaction to produce 2-hydroxy-5-nitro-α-toluenesulfonic acid (III). These observations have been interpreted in terms of the reaction sequence shown in eq 1 which is similar to that postulated to hold for normal ester or amide substrates.[4] In eq 1 E represents the enzyme, S is the sultone, ES is a noncovalent complex of the two species, ES′ is the covalent sulfonyl enzyme intermediate, P is the product acid III, and K_s is the dissociation constant for the ES complex.



I

II



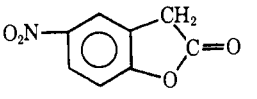
III

$$E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P \qquad (1)$$

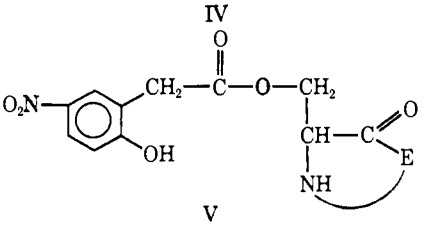
The purpose of the present paper is to report further work that indicates that the reverse of the k_2 step (which we shall call the k_{-2} step) leading to regeneration of the starting ester can be observed in the case of the sulfonyl enzyme II under conditions where the reversion of most acyl enzymes to the corresponding starting esters is negligible.

Results

The nitro-substituted lactone IV has been shown to react with the active site of CT to give the acyl enzyme V according to the reaction sequence of eq 2. In eq 2



IV



V

k_{sp} represents the pseudo-first-order rate constant measured for the nonenzymatic hydrolysis of IV at a given pH in a buffer identical with that used for the enzymatic reaction. In contrast to the general carboxylic ester substrates where the leaving alcohol moieties become part of the solvent when the acyl enzymes are generated, the leaving alcohol in V, the phenolic group, remains in a sterically favorable position for attack on the carbonyl function.[5]

$$E + S \underset{K_s}{\rightleftharpoons} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' \xrightarrow{k_3} E + P \qquad (2)$$
$$S \xrightarrow{k_{sp}} P$$

In view of the results found with the acyl enzyme IV it seemed possible that the scheme of eq 2 would also hold for the reaction of the sultone II with CT. Assuming the establishment of equilibria, the rate of product formation in eq 2 can be expressed by eq 3 where $K_2 = k_{-2}/k_2$.

$$\frac{d(P)}{dt} = k_{sp}[S] + k_3[ES'] = \left[\frac{k_{sp}K_sK_s}{[E]} + k_3\right][ES'] \qquad (3)$$

From eq 3 we may conclude that the formation of the product sulfonic acid III will be a first-order process only if the free enzyme concentration is essentially constant or if $(k_{sp}K_sK_2)/[E] \ll k_3$. Since in our measurements of the kinetics of the production of III we

(1) Predoctoral Fellow of the National Science Foundation.
(2) Fellow of the Alfred P. Sloan Foundation; to whom correspondence should be addressed.
(3) J. H. Heidema and E. T. Kaiser, *J. Amer. Chem. Soc.*, **89**, 460 (1967); **90**, 1860 (1968).
(4) M. L. Bender and F. J. Kézdy, *Annu. Rev. Biochem.*, **34**, 49 (1965).
(5) P. Tobias, J. H. Heidema, K. W. Lo, E. T. Kaiser, and F. J. Kézdy, *J. Amer. Chem. Soc.*, **91**, 202 (1969).

Table I. Formation of 2-Hydroxy-5-nitro-α-toluenesulfonic Acid from 2-Hydroxy-5-nitro-α-tolenesulfonyl-chymotrypsin[a]

E_0, M (initial enzyme concn)	S_0, M (initial sultone concn)	k_{obsd}, sec^{-1}
7.77×10^{-5}	5.03×10^{-5}	6.78×10^{-4}
7.77×10^{-5}	5.03×10^{-5}	6.81×10^{-4}
7.77×10^{-5}	1.01×10^{-5}	6.63×10^{-4}
7.77×10^{-5}	1.01×10^{-5}	6.71×10^{-4}
1.55×10^{-5}	1.01×10^{-5}	6.77×10^{-4}

[a] In 0.05 M phosphate buffer containing 1.64 % CH_3CN, at pH 7.58 and 25.0°.

observed first-order kinetics even when [E] changed appreciably during the course of product formation (see Table I, where k_{obsd} is the rate constant measured for the formation of III) eq 2 can be consistent with our results only if $(k_{sp}K_sK_2/[E]) \ll k_3$. The term $(k_{sp}K_s \cdot K_2/[E])$ may be altered not only by changing [E] but also by changing k_{sp}, the rate constant for solvent-catalyzed or "spontaneous" hydrolysis of the sultone II. Since k_{sp} for sultone II is greater in phosphate buffer than in Tris or Barbital buffer at the same pH, we decided to compare the rate constants for the formation of III from ES′ in these three different kinds of buffers at a given pH. We also checked the possibility that k_{sp} may be appreciably accelerated simply by the presence of chymotryptic protein. (In other words the protein in the solution might catalyze solvolysis of the sultone by a nonenzymatic mechanism analogous to buffer catalysis.) As a model for the chymotryptic protein we used a preparation of α-toluenesulfonyl-CT. The results of these experiments are summarized in Table II.

Table II. Effect of Changes in the Rate of Hydrolysis of 2-Hydroxy-5-nitro-α-toluenesulfonic Acid Sultone upon the Rate of Formation of Sulfonic Acid III from 2-Hydroxy-5-nitro-α-toluenesulfonyl-chymotrypsin (II)

Expt no.	Reaction[a]	Buffer[b]	$k \times 10^4$, sec^{-1}	pH
1	Formation of III from II	P	8.27	7.26
2	Hydrolysis	P	47.1	7.27
3	Formation of III from II	T	8.23	7.23
4	Hydrolysis	T	6.13	7.23
5	Formation of III from II	B	8.68	7.20
6	Hydrolysis	B	2.35	7.23
7	Hydrolysis	B′	3.32	7.28
8	Hydrolysis	B′	3.34	7.28

[a] All reaction solutions were initially 6.7×10^{-5} M in sultone I. In the experiments measuring the rate of formation of the sulfonic acid III from the sulfonyl enzyme II the buffer was made 9.25×10^{-5} M in CT before sultone I was added. [b] P is 0.100 M (total) phosphate, T is 0.100 M (total) Tris, B is 0.040 M (total) Barbital, B′ is B containing 1.00×10^{-4} M α-toluenesulfonyl-CT. For all buffers I = 0.40 (NaCl) and all contained 2.2% (v/v) CH_3CN.

From the data of Table II we can see that the rate constants observed for the formation of III by the decomposition of the sulfonyl enzyme II are not noticeably affected by a 14-fold change in k_{sp}. We may also conclude that 10^{-4} M chymotryptic protein only accelerates k_{sp} very slightly. Furthermore, our results indicate that the k_3 step of eq 2 is the principal pathway for the production of the sulfonic acid III from II under the conditions of the experiments which have been discussed.

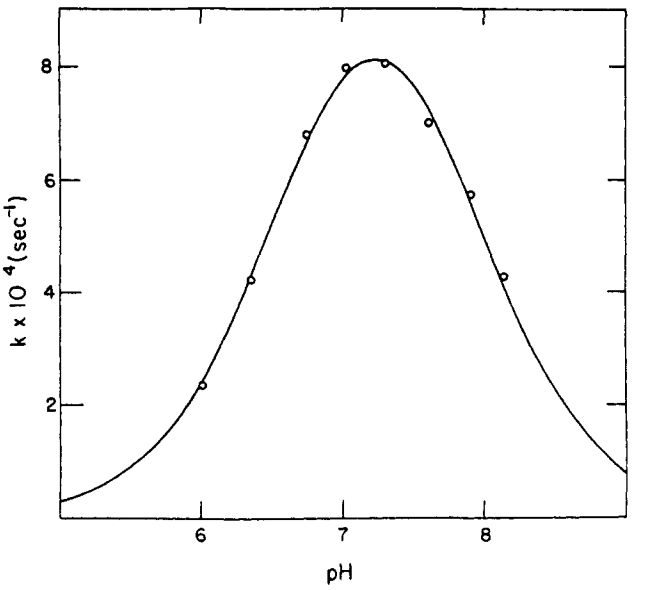


Figure 1. pH profile for the formation of 2-hydroxy-5-nitro-α-toluenesulfonic acid (III) from 2-hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin (II) in 0.10 M phosphate buffers adjusted to I = 0.40 with NaCl at 25.0°. The curve is a theoretical one for pK_M = 6.59, pK_N = 7.87, and k(lim) = 11.8×10^{-4} sec^{-1} (a computer-calculated, least-squares fit; see ref 6). The solutions contained 0.94% CH_3CN.

In our earlier study we found that the rate constants measured for the formation of the sulfonic acid III from II exhibited a bell-shaped pH dependence.[3] Under the conditions employed the ionic strength varied from I = 0.05 to 0.15 over the pH range examined. We discovered later that the rate of production of the sulfonic acid III from II depends somewhat upon the ionic strength of the solution. Therefore, the pH–rate profile for the enzymatic formation of III has been redetermined, this time with phosphate buffers adjusted to I = 0.40 with NaCl. The pH–rate profile obtained is shown in Figure 1. A computer-calculated least-squares fit to the data gave pM = 6.59 and pN = 7.87, where M refers to the observed ionization constant on the acidic side of the profile and N refers to that on the alkaline side.[6]

An interpretation of the bell-shaped pH dependence of the rate of formation of III from II which is consistent with the results of a spectral titration of the nitrophenol chromophore of II has been presented already in terms of the scheme shown in eq 4.[3] The spectral titration results showed that the ionization of the phenolic proton in the sulfonyl enzyme II is influenced by another ionization of a nearby group on the protein occurring at a similar pH.[3] The group perturbing the ionization of the phenol function is most likely the imidazole ring of histidine-57 of the active site. In eq 4, HONH represents the sulfonyl enzyme protonated at both the imidazole and phenol groups, ON is the sulfonyl enzyme with both groups deprotonated, and ONH and HON are singly protonated species at the imidazole and phenol, respectively. By the procedure outlined earlier[3] we have calculated the following pK values for the groups in eq 4: pK_1 = 6.75, pK_2 = 6.76, pK_3 = 7.57, and pK_4 = 7.56.[7]

(6) A computer program written by Dr. P. L. Hall was used. See: P. L. Hall, Ph.D. Thesis, University of Chicago, 1967.

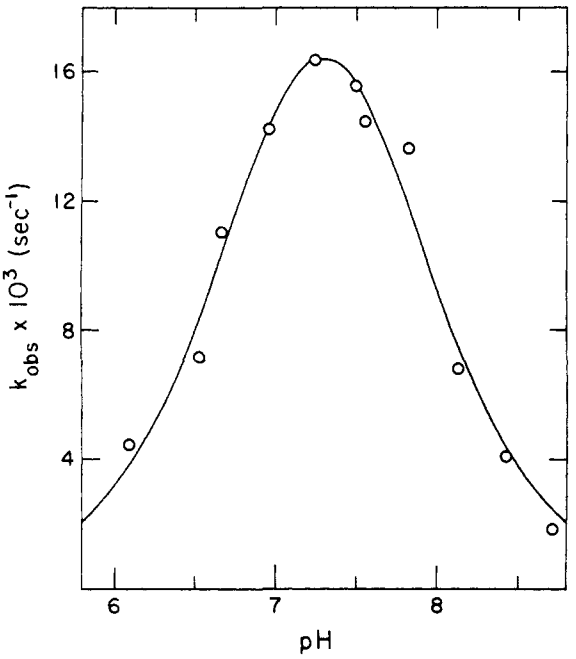


Figure 2. pH profile for the desulfonylation of 2-hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin (a combination of steps k_{-2} and k_3 of eq 2) at 25.0°. The curve is a theoretical one for pK_M = 6.96, pK_N = 7.64, and k_{obsd}(lim) = 4.54 × 10^{-2} sec^{-1} (a computer-calculated least-squares fit). The buffers were of ionic strength 0.40 and contained 2.4% CH_3CN.

$$\begin{array}{ccc} \text{HONH} & \overset{K_1}{\rightleftharpoons} & \text{ONH} \\ \downarrow\uparrow K_2 & & \downarrow\uparrow K_3 \\ \text{HON} & \underset{K_4}{\rightleftharpoons} & \text{ON} \end{array} \qquad (4)$$

We have hypothesized that the reactive form of the sulfonyl enzyme leading to the production of the sulfonic acid III is species HON of eq 4.[3] In other words the sulfonyl enzyme has the imidazole ring of histidine-57 unprotonated and the phenolic hydroxyl protonated in the state giving rise to the production of III. The constants M and N obtained from the data of Figure 1 are related to the ionization constants of eq 4 by eq 5 and 6, respectively.[3] The agreement between the ion-

$$M = K_1 + K_2 \qquad (5)$$

$$N = K_4/[1 + (K_1/K_2)] \qquad (6)$$

ization constants calculated from the titration of the phenolic hydroxylic group in 2-hydroxy-5-nitro-α-toluenesulfonyl-α-CT (II) and those found from the kinetics of the production of sulfonic acid from II is good.[8]

None of the evidence presented so far based on observations of the production of III supports the inclusion of the k_{-2} step of eq 2 in the desulfonylation of 2-hydroxy-5-nitro-α-toluenesulfonyl-α-CT (II). However, this may simply mean that although eq 2 correctly describes the reaction which the sultone I undergoes with CT, at any appreciable free enzyme concentration [E], the value of [S] ≪ [ES']. In other words under the experimental conditions employed in the measurements recorded in Table I, the equilibrium in eq 2 lies far to the right. If this is the correct explanation for the above observations, the decomposition of the sulfonyl enzyme under conditions where free enzyme is almost completely absent from the reaction medium should result, according to eq 2, in an observed rate constant for the decomposition of the sulfonyl enzyme, k_{obsd}, expressed by eq 7. Such conditions exist when the desulfonylation of the sulfonyl enzyme is followed by a rate-assay technique using a specific ester substrate for CT. With such a technique the initial sulfonyl enzyme concentration can be as low as 10^{-7} M, and the released free enzyme formed by desulfonylation of II can be essentially saturated with the specific ester substrate. A convenient specific ester substrate which can be employed to assay CT is N-acetyl-L-tryptophan methyl ester.[9] The hydrolysis of this compound may

$$k_{obsd} = k_{-2} + k_3 \qquad (7)$$

be followed spectrophotometrically by observing the absorbance increase at 300 mμ. We therefore used the rate-assay technique with this substrate to measure the rate of decomposition of 2-hydroxy-5-nitro-α-toluenesulfonyl-α-CT (II).

The experimental procedure used was to add a dilute solution of II to a buffered solution of 10^{-3} M N-acetyl-L-tryptophan methyl ester at the desired pH. From the change in absorbance with time, $\Delta A/\Delta t$, at 300 mμ it was possible to determine a first-order rate constant for the decomposition of the sulfonyl enzyme II. At a given pH this observed rate constant was considerably larger than the rate constant measured for the formation of the product sulfonic acid III. This finding supports the hypothesis that in analogy to our observations with the acyl enzyme the k_{-2} step of eq 2 is important in the decomposition of the sulfonyl enzyme in addition to the k_3 step which gives rise to the sulfonic acid. In other words our rate assay measurements provide evidence that the phenolic hydroxyl group in 2-hydroxy-5-nitro-α-toluenesulfonyl-α-CT participates in an intramolecular nucleophilic displacement reaction at the sulfonyl group leading to the regeneration of the starting sultone I.

The pH dependence of the k_{obsd} values measured by the rate-assay technique with N-acetyl-L-tryptophan methyl ester was determined. As indicated in Figure 2, a bell-shaped pH profile was obtained. A computer-calculated least-squares fit to the data gives a maximum rate at pH 7.30 with pM = 6.96 and N = 7.64.[6] Since the rate constants measured here were about 20 times greater than those measured under comparable conditions for the production of the sulfonic acid III, we conclude that $k_{obsd} \approx k_{-2}$ (see eq 7), and therefore Figure 2 illustrates the pH dependence of k_{-2}. From this pH dependence it can be seen that two ionizing groups in II must be involved in the k_{-2} step of eq 2. A comparison of the computer-calculated values for M and N from the data of Figure 1 (representing the k_3 step) and those of Figure 2 (representing the k_{-2} step) reveals a reasonably close correspondence in the param-

(7) On the basis of additional results these pK values have been revised somewhat from those given previously in ref 3. We reiterate here that we do not intend to imply that eq 4 uniquely accounts for the ionization behavior of the nitrophenol chromophore. However, it represents a reasonable postulate.

(8) The value of pM determined from spectral titration is 6.46 and that of pN is 7.86. See: J. H. Heidema, Ph.D. Thesis, University of Chicago, 1969.

(9) B. Zerner, R. P. M. Bond, and M. L. Bender, *J. Amer. Chem. Soc.*, 86, 3674 (1964).

eters measured from the two figures especially when the standard deviations are considered.[10] The standard deviations for the data of Figure 2 were particularly large. The technique by which these data were obtained permitted considerable experimental error.

An effect other than the intramolecular recyclization reaction (step k_{-2} of eq 2) of the sulfonyl enzyme II regenerating the sultone I could conceivably have caused the accelerated desulfonylation rates observed under the rate-assay conditions. The deacylation of acetyl-CT is accelerated by the presence of indole[11] or *N*-acetyl-L-tyrosine ethyl ester.[12] This occurs presumably because the acetyl enzyme can bind the activator, and this bound complex can deacylate faster than the uncomplexed acetyl-CT. The accelerated desulfonylation rate we have observed in the presence of *N*-acetyl-L-tryptophan methyl ester might have been caused by a similar phenomenon. That is, the sulfonyl enzyme II might be capable of binding the ester substrate, and this resulting complex might desulfonylate faster than the uncomplexed sulfonyl enzyme.[13] However, this interpretation of the rate-assay results requires that the observed rate constant should increase with an increase in the concentration of the rate-assay substrate because there should be an increase in the ratio of complexed to uncomplexed sulfonyl enzyme.[14] In one experiment, therefore, the rate of desulfonylation of II was measured at two different concentrations of the rate-assay substrate with all other variables (pH, temperature, and per cent organic solvent) being held constant. The calculated rate constant at 2.00×10^{-3} *M* substrate (0.0107 sec^{-1}) was the same as it was at 1.00×10^{-3} *M* substrate (0.0115 sec^{-1}). A reaction involving intramolecular attack of the phenolic hydroxyl group in the sulfonyl enzyme II to regenerate the starting sultone I is therefore the most probable explanation accounting for the rapid rate of desulfonylation observed at low sulfonyl enzyme concentrations in the presence of a rate-assay substrate.

In all of the kinetic studies in which the rate of production of the sulfonic acid III from the sulfonyl enzyme II was directly measured, the observed rates were strictly first order, with changes in [E] or k_{sp} having no effect on the observed rate constants. A question can be posed whether eq 2 and its rate constants as determined in our various kinetic studies would predict this behavior. According to eq 2 and 3, the production of sulfonic acid III can be first order only if $(k_{sp}K_sK_2)/[E] \ll k_3$. We will now calculate whether the kinetic parameters we have found meet this condition. For the purposes of this calculation we will employ data obtained at pH 7.27. The kinetic parameters of eq 2 at pH 7.27 are: $k_2 = 4.71 \times 10^{-3}$ sec^{-1}, $k_2/K_s = 1.9 \times 10^5$ M^{-1} sec^{-1}, $k_{-2} = 1.56 \times 10^{-2}$ sec^{-1}, and $k_3 = 8.1 \times 10^{-4}$ sec^{-1}.[8] Therefore $k_{sp}K_sK_2/[E] = (k_{sp}(K_s/k_2)k_{-2})/[E] = (1/[E]) \times 3.9 \times 10^{-10}$ *M* sec^{-1}. Under the conditions used to collect the data in Figure 1, [E] is always $\geq 2 \times 10^{-5}$ *M*. Hence $k_{sp}K_sK_2/[E] \leq (3.9 \times 10^{-10}$ *M* sec$^{-1})/(2 \times 10^{-5}$ *M*$) = 2.0 \times 10^{-5}$ sec^{-1}. If $k_{sp}K_sK_2/[E]$ is always $\geq 0.20 \times 10^{-4}$ sec^{-1} while $k_3 = 8.1 \times 10^{-4}$ sec^{-1}, changes in [E] cannot affect significantly the observed rate constant for sulfonic acid production and the reaction will appear to be strictly first order. Desulfonylation of 2-hydroxy-5-nitro-α-toluenesulfonyl-α-CT *via* the sequence of reactions shown in eq 2 is consistent thus with all the presently available data.[15]

Discussion

Having provided evidence for the scheme of eq 2 as the sequence for the reaction of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I) with α-chymotrypsin, we can now pose the problem whether pentacoordinate intermediates are formed during sulfonylation or desulfonylation. A number of arguments including ones based on the principle of microscopic reversibility have been advanced to support the postulation of tetrahedral addition intermediates in the acylation and deacylation reactions undergone by carboxylic esters with α-CT.[16] In the case of carboxylic esters, of course, a great deal of evidence exists that tetrahedral intermediates are formed in nonenzymatic solvolysis reactions.[17] However, in the nonenzymatic solvolysis reactions of sulfonate esters there is no compelling evidence for the postulation of pentacoordinate addition intermediates.[18] There is no strong precedent, thus, in the chemistry of sulfonate esters on which to base the postulation of such intermediates in the chymotrypsin-catalyzed reaction of the sultone II. Nevertheless, our observations that the reaction sequence of eq 2 appears to apply both to the reaction of the cyclic carboxylic ester IV and to that

(10) The computer-calculated least-squares fit to the data gives the following values for the parameters and their standard deviations: for the data of Figure 1, $M = 257 \pm 25$, $N = 13.4 \pm 1.3$; and for the data of Figure 2, $M = 111 \pm 77$ and $N = 23.1 \pm 16.2$ (all numbers multiplied by 10^9).

(11) R. J. Foster, *J. Biol. Chem.*, **236**, 2461 (1961).

(12) M. L. Bender, F. J. Kézdy, and C. R. Gunter, *J. Amer. Chem. Soc.*, **86**, 3714 (1964).

(13) Admittedly, one might think that the binding of another molecule to the CT active site is less likely in the presence of the 2-hydroxy-5-nitro-α-toluenesulfonyl group than in the presence of the much smaller acetyl group.

(14) This statement assumes that the concentration of the rate assay substrate is such that the sulfonyl enzyme would *not* be saturated by it.

(15) Some preliminary experiments have been performed with the irreversible inhibitor α-toluenesulfonyl fluoride (unpublished results, Dr. S. F. Bosen) and with the active-site titrant cinnamoylimidazole in attempts to detect the k_{-2} step of eq 2 by measuring the decrease in absorbance at 390 mμ which should occur as 2-hydroxy-5-nitro-α-toluenesulfonyl-α-CT undergoes the recyclization reaction to form the sultone I. In order to quantitatively measure the rate of the k_{-2} step in this way it would be necessary to employ a reagent which quantitatively scavenges free enzyme as it is formed by desulfonylation. Furthermore, a sufficient initial concentration of sulfonyl enzyme must be present to allow accurate measurement of the disappearance of the 390-mμ phenolate absorbance. Finally, the scavenging reagent employed should not absorb in an area of the ultraviolet–visible spectrum which would interfere with measurements on the disappearance of the absorption due to the phenol chromophore of the sulfonyl enzyme. Although we have been able to meet some of these conditions in our preliminary experiments we have not as yet found experimental conditions over a wide pH range under which all of these conditions apply. Nevertheless, the preliminary experiments we have performed trapping free enzyme with α-toluenesulfonyl fluoride or cinnamoylimidazole are in at least qualitative agreement with the rate-assay results which have been discussed. For example, an experiment was performed in which sulfonyl enzyme II was generated by treating 2.48×10^{-5} *M* CT with 2.45×10^{-5} *M* sultone I in a pH 7.15 saturated barbital buffer in the presence of 0.4 *M* NaCl. Then 3.78×10^{-4} *M* α-toluenesulfonyl fluoride was added. The total volume of CH_3CN present was 3.9%. Observation of the decrease in absorption at 390 mμ which occurred on addition of α-toluenesulfonyl fluoride gave a first-order rate constant of 1.38×10^{-2} sec^{-1} (results of Dr. S. F. Bosen). This rate constant agreed quite well with the value of k_{-2} which we measured under similar reaction conditions using the rate-assay technique ($k_{-2} = 1.5 \times 10^{-2}$ sec^{-1}) although even in this instance calculations can be performed which show that α-toluenesulfonyl fluoride does not quantitatively trap free enzyme as it is formed.

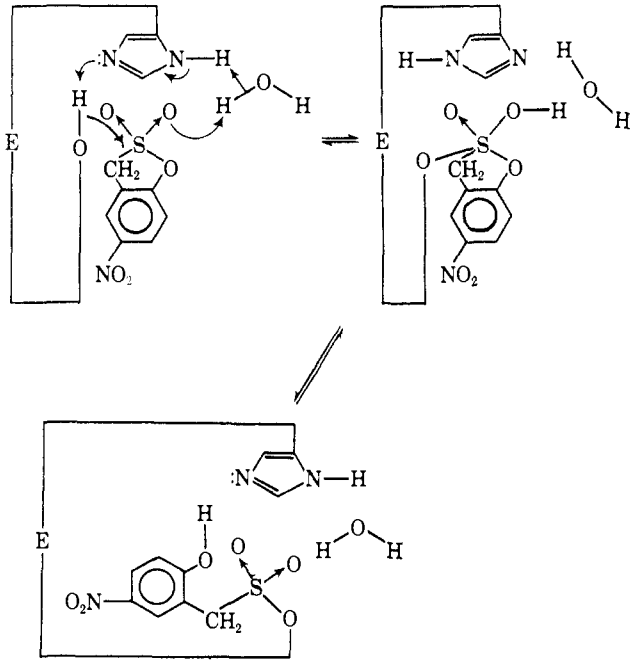
(16) For a further discussion see: W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, pp 218–226.

(17) M. L. Bender, *Chem. Rev.*, **60**, 53 (1960).

(18) E. T. Kaiser, *Accounts Chem. Res.*, **3**, 145 (1970).

of the cyclic sulfonate ester I with α-CT leads us to suggest that the stepwise processes by which these two types of esters react with the enzyme may be analogous. On the basis of such a hypothesis the mechanism of Scheme I can be written in the case of the sulfonyl enzyme for the K_s and K_2 equilibria of eq 2.[19] In this mechanism the attack of the serine hydroxyl group on the sulfonyl sulfur atom of the substrate is aided by both general acid and general base catalysis by the imidazole group of histidine-57. A molecule of water serves as the proton transfer agent for the general acid catalyzed part of the reaction. In a symmetrical manner the decomposition of the pentacoordinate intermediate occurs *via* a general acid–general base catalyzed process, a molecule of water serving as the proton transfer agent for the general base catalyzed part of the reaction. The mechanism of Scheme I is an attractive one for the rereaction of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone with α-chymotrypsin. It is consistent with the

Scheme I



kinetic and spectral data discussed in this paper as well as the structural and mechanistic information on α-chymotrypsin presently available in the literature and can serve as a working hypothesis for the future.

Experimental Section

Materials. The determination of the normality of enzyme solutions, the preparation of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone, and the preparation of reaction solutions were done as previously described.[3]

Preparation of *N*-Acetyl-L-tryptophan Methyl Ester. *N*-Acetyl-L-tryptophan was a product of Mann Research Laboratories. A solution of diazomethane, 0.6 *M* in ethyl ether, was prepared according to the procedure of Arndt.[20] To 1.00 g (0.0406 mol) of *N*-acetyl-L-tryptophan dissolved in about 50 ml of methanol was added 10 ml of the diazomethane solution. A few drops of acetic acid were then added to decompose excess diazomethane, and the solvent was removed on a rotary evaporator. The gummy, light brown residue (smelling strongly of acetic acid) was left exposed to air for a few days whereupon the residue became crystalline. It was then dissolved in boiling ethyl acetate and treated with decolorizing carbon. The product which crystallized from concentrated ethyl acetate was twice recrystallized from chloroform–cyclohexane to give pure white needles, mp 155–156.5° (lit.[9] mp 154–155°).

Kinetics of Formation of Sulfonic Acid from the Sulfonyl Enzyme II. These kinetic measurements were carried out as described previously.[3] However, the results discussed in the present paper were obtained in 0.10 *M* phosphate buffer adjusted to $I = 0.40$ with NaCl at 25.0°.

Rate-Assay Measurements to Determine the pH Dependence of the Desulfonylation of the Sulfonyl Enzyme II. The buffers employed across the pH range were either 0.100 *M* (total) in phosphate or 0.100 *M* (total) in Tris with ionic strengths adjusted to 0.40 with NaCl. CT solutions were inhibited at concentrations varying from 5 to 20 $\times 10^{-6}$ *M* in the same buffers used for the assays by the addition of an approximately threefold (molar) excess of sultone I in acetonitrile. The rate assays were initiated 1–2 min after sultone addition. The enzyme was assayed with *N*-acetyl-L-tryptophan methyl ester in a substrate concentration range where the rate of hydrolysis catalyzed by the uninhibited enzyme is essentially independent of substrate concentration. For the assay 50 μl of a 4.00 $\times 10^{-2}$ *M* solution of the substrate in acetonitrile was added to 2.00 ml of the desired buffer, and the resultant solution was allowed to equilibrate in the thermostated cell compartment of a Cary 15 spectrophotometer for about 15 min. Then 20 μl of freshly inhibited enzyme solution of the appropriate concentration was added, and the absorbance change with time was recorded at 300.0 mμ[21] using a slide wire producing a full-scale pen deflection for an absorbance change of 0.1 unit. The total enyzme concentration during the assays was therefore between 5 and 20 $\times 10^{-8}$ *M* (the higher concentrations were employed at the lower pH values), and the initial concentration of *N*-acetyl-L-tryptophan methyl ester was 1.0 $\times 10^{-3}$ *M*. In the assays testing the effect of changes in the initial substrate concentration upon the observed reactivation rate constants, one assay was performed using 100 μl of substrate solution, the other using a mixture of 50 μl of acetonitrile and 50 μl of substrate solution.

Reactivation of the inhibited enzyme under these conditions was relatively fast, with half-lives as low as 40 sec being observed. The rate-assay conditions were designed therefore so that reactivation was essentially complete before too much of the assay substrate was consumed.[22] Since the change in absorbance with time, $\Delta A/\Delta t$, at any substrate concentration was proportional to the concentration of active CT, the first-order rate constant for reactivation of the inhibited enzyme could be determined from the rate of change in $\Delta A/\Delta t$ with time. Thus, when $\Delta A/\Delta t$ at time t was subtracted from $\Delta A/\Delta t$ for the completely reactive enzyme and this difference (ΔS) was plotted *vs.* time on semilogarithmic graph paper, the points defined a straight line. The straight line best conforming to the experimental points was drawn, and from two points on this line the first-order rate constant k_{obsd} (see eq 7) for reactivation was determined using eq 8. In some of the assays reactivation was not complete before the substrate concentration fell below permissible limits. In these cases $\Delta A/\Delta t$ for the 100% reactivated enzyme was estimated.

$$k_{obsd} = 2.303/(t_2 - t_1) \log \Delta S_1/\Delta S_2 \qquad (8)$$

There was much more uncertainty in the experimental points for these reactivation kinetics than in the rate measurements in which the production of 2-hydroxy-5-nitro-α-toluenesulfonic acid was followed directly. The rate constants for the kinetics of reactivation of the inhibited enzyme were subject thus to a much greater experimental error. This fact is made evident by the greater scatter of the points and the greater standard deviations in the

(19) This mechanism is similar to a mechanism proposed by M. L. Bender and F. J. Kézdy, *J. Amer. Chem. Soc.*, **86**, 3704 (1964), for carboxylic ester substrates. (See eq 10 of their paper.) Our Scheme I is actually directly analogous to the modified version of the Bender–Kézdy mechanism presented by H. R. Mahler and E. H. Cordes, "Biological Chemistry," Harper and Row, New York, N. Y., 1966, p 312.

(20) F. Arndt in "Organic Syntheses," Coll. Vol. II, A. H. Blatt, Ed., Wiley, New York, N. Y., 1943, p 165.

(21) The hydrolysis of *N*-acetyl-L-tryptophan methyl ester to *N*-acetyl-L-tryptophan and methanol in water solutions occurs with a $\Delta\epsilon = +240$ at 300 mμ.[9]

(22) The experiments were designed so that reactivation was complete before the substrate concentration dropped below 6×10^{-4} *M*. Below this concentration the rate of the CT-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester is noticeably dependent upon the substrate concentration.

computer-calculated parameters for the pH-rate profile in Figure 2 as compared to those for the profile of Figure 1.

Conclusion

In agreement with model system studies[23] the present work demonstrates that a newly introduced intramolecular nucleophile in an enzyme is far more effective than the external nucleophile water. Specifically, α-toluenesulfonyl-α-CT which does not possess such an intramolecular nucleophile is unable to desulfonylate in the pH range near neutrality[24] through the attack of water catalyzed by the enzyme. However, the enzyme-catalyzed attack of the phenolic hydroxyl on the sulfonyl group in 2-hydroxy-5-nitro-α-toluenesulfonyl-α-CT is very effective. Thus, the enzyme appears to be able to utilize the proximity effect of the phenolic hydroxyl group in desulfonylation thereby greatly favoring kinetic control (re-formation of the sultone) rather than thermodynamic control (formation of sulfonic acid). By analogy, this enzyme model system lends credit to the pathway proposed for the re-formation of virgin soybean inhibitor from the acyl-trypsin produced by the interaction of trypsin with soybean trypsin inhibitor.[25] Therefore, it appears that the most powerful naturally occurring serine proteinase inhibitors probably form acyl-enzymes, but in order to maintain the efficiency of the inhibitor the destruction of the inhibitor by normal hydrolysis should be avoided. In other words, the re-formation of the native inhibitor by an intramolecular nucleophilic reaction should be favored. Thus, we suggest that the powerful naturally occurring serine proteinase inhibitors owe their efficiency to the presence of covalently bound leaving groups in the acyl enzymes produced by the reaction of the inhibitors with the enzymes.

Acknowledgment. This research was supported in part by a grant from the National Institute of General Medical Sciences. We wish to thank Professor F. J. Kézdy for helpful discussions.

(23) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, pp 8-35.
(24) D. E. Fahrney, Ph.D. Thesis, Columbia University, 1963.
(25) W. R. Finkenstadt and M. Laskowski, Jr., *J. Biol. Chem.*, **242**, 771 (1967); M. Laskowski, Jr., Abstracts, International Union of Biochemistry Symposium on Structure-Function Relationships of Proteolytic Enzymes, Copenhagen, June 16-18, 1969; R. W. Sealock and M. Laskowski, Jr., *Biochemistry*, **8**, 3703 (1969).

Communications to the Editor

Direct Reaction of Triphenyl Phosphite Ozonide with *cis*- and *trans*-Diethoxyethylenes[1]

Sir:

Triphenyl phosphite ozonide (**2**), formed at −78° by the addition of ozone to triphenyl phosphite[2] (**1**), decomposes spontaneously at −17° ($k_1 = 1.58 \times 10^{-3}$ sec^{-1} at −17° in CH_2Cl_2)[3a] to triphenyl phosphate (**3**) and singlet oxygen (eq 1 and 2).[3a-c] The singlet oxygen

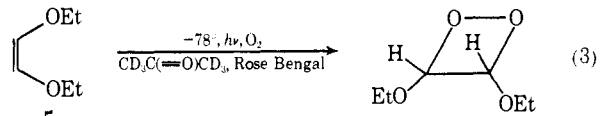
$$\underset{\mathbf{1}}{(C_6H_5O)_3P} + O_3 \xrightarrow{-78^\circ} \underset{\mathbf{2}}{(C_6H_5O)_3PO_3} \qquad (1)$$

$$\underset{\mathbf{2}}{(C_6H_5O)_3PO_3} \xrightarrow{-17^\circ} \underset{\mathbf{3}}{(C_6H_5O)_3PO} + {}^1O_2 \qquad (2)$$

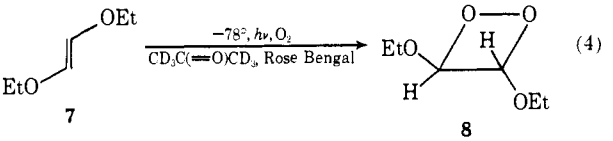
produced in this decomposition can be trapped by acceptors in solution to yield the characteristic singlet oxygen products.[3a-c,4a]

Recently, Bartlett and Mendenhall[4a] have shown that the ozonide **2** also undergoes a bimolecular reaction with tetramethylethylene (**4**) at −70°, at which temperature **2** is indefinitely stable, to form the same allylic hydroperoxide produced by photooxidation of **4**.[5] The corresponding reaction of the ozonide **2** with most dienes and substituted anthracenes either does not occur or is imperceptibly slow.[4a,b] These authors have also established that this reaction is not the result of a catalytic decomposition of the ozonide **2** by the olefin **4**.

We now report that triphenyl phosphite ozonide (**2**) is able to imitate another reaction of singlet oxygen, the 1,2 cycloaddition to vinylene diethers to yield 1,2-dioxetanes. Singlet oxygen produced photochemically adds stereospecifically to *cis*- and *trans*-diethoxyethylenes (**5** and **7**) to give the dioxetanes **6** and **8**, respectively.[6a,b] The ozonide **2** also yields **6** and **8** from **5** and **7**, but in its stereochemical course the reaction contrasts sharply with the stereospecific behavior of free singlet oxygen. The bimolecular reaction at −78°, in tri-



5 6 (3)



7 8 (4)

chlorofluoromethane, of the ozonide **2** with **5** and with **7** yields the same mixture of the two isomeric dioxetanes

(1) Presented in part at the 160th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1970.
(2) Q. E. Thompson, *J. Amer. Chem. Soc.*, **83**, 845 (1961).
(3) (a) R. W. Murray and M. L. Kaplan, *ibid.*, **91**, 5358 (1969); (b) R. W. Murray and M. L. Kaplan, *ibid.*, **90**, 527 (1968); (c) E. Wasserman, R. W. Murray, M. L. Kaplan, and W. A. Yager, *ibid.*, **90**, 4160 (1968).
(4) (a) P. D. Bartlett and G. D. Mendenhall, *ibid.*, **92**, 210 (1970). (b) 2,5-Dimethylfuran reacts slowly with **2** at −70° to give a mixture of products.[4a]
(5) C. S. Foote, *Accounts Chem. Res.*, **1**, 104 (1968), and references therein.
(6) (a) P. D. Bartlett and A. P. Schaap, *J. Amer. Chem. Soc.*, **92**, 3223 (1970). (b) Mazur and Foote have reported the addition of singlet oxygen to tetramethoxyethylene: S. Mazur and C. S. Foote, *ibid.*, **92**, 3225 (1970).