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The Reaction of a Cyclic Sulfonate Ester with the Sulfhydryl Proteolytic Enzyme Papain

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The reactive aromatic 5-membered cyclic sulfonate ester 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I) undergoes facile papain-catalyzed hydrolysis. This finding is remarkable since it implies that the intermediate sulfonyl-enzyme, which is presumably a thiolsulfonate species, is labile while the corresponding unsubstituted sulfonyl-enzyme, α-toluenesulfonyl-papain, is very stable under the same conditions. As has been suggested in the case of the chymotrypsin-catalyzed hydrolysis of I, the phenolic hydroxyl group in 2-hydroxy-5-nitro-α-toluenesulfonyl-papain may be acting as an intramolecular general acid catalyst, assisting the hydrolytic breakdown of the sulfonyl-enzyme. A kinetic analysis has revealed that concurrently with the process of sultone hydrolysis, papain is modified by the sultone I, giving an inactive enzyme. Apparently, the intermediate sulfonyl-enzyme decomposes about 2% of the time to form the inactive species, which contains a covalently bound *p*-nitrophenol chromophore, rather than to give the 2-hydroxy-5-nitro-α-toluenesulfonate anion. The nature of the inactivated enzyme is currently under investigation.

INTRODUCTION

Of the wide variety of chemical techniques used to probe the active sites of enzymes, one of the most interesting has been the generation of new internal nucleophiles *via* modification of the enzymes with labile cyclic esters. Recent work on the reactions of cyclic sulfonate esters, or sultones, such as I, with α-chymotrypsin has been reviewed (*1*). A comparison of the reactivity of the sulfonyl-enzyme II, produced from the sulfonylation of α-chymotrypsin by I, with that of the sulfonyl-enzyme III, formed from the reaction of α-toluenesulfonyl fluoride with the enzyme, is particularly interesting. The latter sulfonyl enzyme is completely inactive over the pH range 3 to 8.5 under the usual conditions of assay (*2*). In contrast, II desulfonylates readily at neutral pH to yield active α-chymotrypsin and the sulfonate anion IV (*3*).

The sequence by which I reacts with α-chymotrypsin can be represented by Eq. 1, an essentially similar pathway to that followed by carboxylic ester substrates (*4–6*). There is, however, one important difference in the pathways for the desulfonylation of II and the deacylation of the usual acyl-α-chymotrypsin intermediate. The k_{-2} step in Eq. 1,

$$\mathrm{I} + \mathrm{HO{-}CH_2{-}Enz} \underset{K_s}{\rightleftharpoons} \mathrm{I \cdot HO{-}CH_2{-}Enz} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{II} \xrightarrow{k_3} \mathrm{IV} + \mathrm{HO{-}CH_2{-}Enz} \quad (1)$$

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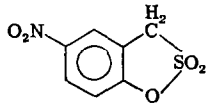
corresponding to the regeneration of the starting ester, is significant for the sulfonyl-enzyme II under conditions where this pathway for most acyl-enzymes is negligible relative to the step in which free carboxylic acid is formed. Since significant k_{-2} steps have been observed in the enzymatic reactions of the related 5-membered cyclic esters V



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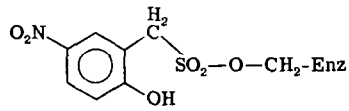

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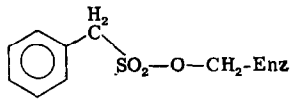

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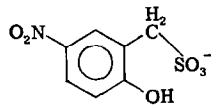
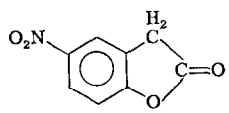


(IV)

(*7*) and VI (*8*), but not in the case of 6-membered rings (*7*), it is likely that the recyclization reaction, a thermodynamically unfavorable process (*8*, *9*), occurs because the proximity of the phenolic hydroxyl group in species like II facilitates attack by it relative to attack by the solvent.



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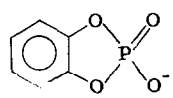

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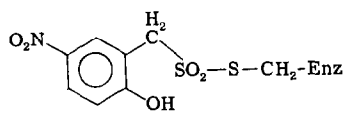
It was of inherent interest to extend these results to another class of enzymes. Papain is a plant proteinase which, like α-chymotrypsin and other serine proteinases, catalyzes the hydrolysis of a variety of substrates, including carboxylic esters and amides (*10*). It differs, however, in that the acyl receptor is a free sulfhydryl of cysteine (*11*) rather than



(VII)

an hydroxyl. If papain were to react with a compound such as I, the presumed intermediate would be the thiolsulfonate VII. Since thiolsulfonates exhibit chemical properties completely different from their oxygen analogs (*12*), it was expected that this enzymatic reaction might show unusual behavior. Such indeed proved to be the case.

RESULTS

A cursory examination revealed that the hydrolysis of the nitro-substituted sultone I is catalyzed by papain,[2] and that under substrate-in-excess conditions the "turnover" process observed cannot be described satisfactorily in terms of the usual pathway for papain catalysis (*13*) involving two catalytic steps. Such a pathway predicts kinetic

[2] Preliminary experiments were performed by Dr. M. Iwatsuru.

behavior of the normal Michaelis–Menten type when $S \gg E$. However, a plot of the rate of the enzyme-catalyzed hydrolysis reaction *vs* the concentration of the nitro-substituted sultone in a single reaction shows the unusual shape illustrated in Fig. 1. The non-

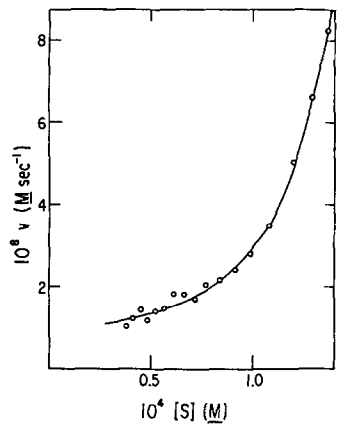


FIG. 1. The rate of the papain-catalyzed hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone as a function of substrate concentration in a single reaction is illustrated. The medium is 0.05 *M* acetate buffer, containing 0.005 *M* EDTA and 0.93% v/v CH_3CN, pH 5.20, $\mu = 0.10$, and $[E]_0 = 2.4 \times 10^{-6}$ *M*.

linearity of a conventional double reciprocal plot is shown in Fig. 2. Since separate experiments showed that added IV had no effect on the rate of reaction of I, or in other words, product inhibition does not occur, it seems reasonable to conclude that concurrently with the process of sultone hydrolysis, papain undergoes a modification to an inactive form.

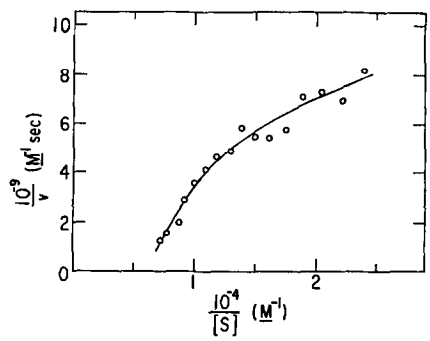


FIG. 2. The data of Fig. 1 are plotted in standard double reciprocal form.

This conclusion is confirmed by several experiments. When a 70-fold excess of I is allowed to hydrolyze completely in the presence of papain, the final activity of the enzyme is drastically reduced. The reduction of activity is parallel when measured either by a standard assay with *p*-nitrophenyl *N*-benzyloxycarbonylglycinate (Z–GlyPNP) (*14*), by an active site titration with 4-hydroxy-3-nitrophenacyl bromide (*15*,) by sulfhydryl titration with 5,5′-dithiobis (2-nitrobenzoic acid) (*16*), or by reactivity toward the nitro-substituted sultone itself. These results are presented in Table 1.

The properties of the inactivated enzyme have been studied subsequent to gel filtration. The most striking observation made is that a solution of the inactive enzyme produced is distinctly yellow colored, showing, when the pH is 6 or above, in addition to the usual protein chromophore with $\lambda_{max} = 279$ nm, an absorption with $\lambda_{max} = 408$ nm,

TABLE 1

ASSAYS OF PAPAIN INACTIVATED BY TREATMENT WITH 2-HYDROXY-5-NITRO-α-TOLUENESULFONIC ACID SULTONE IN 70-FOLD EXCESS[a]

Substrate	% Activity remaining After 5 min	After 3 hr
I	32 ± 4	<2
Z-GlyPNP	36.6 ± 0.8	<0.5
DTNB[b]	39.8 ± 1.0	<0.5
HNPB[c]	31 ± 5	<3

[a] The inactivation was carried out at pH 5.20 in 0.05 *M* acetate buffer at 25°C, containing 0.005 *M* EDTA and 1.9% v/v CH_3CN, with $\mu = 0.1$; $[E]_0 = 3.0 \times 10^{-5}$ *M*, $[S]_0 = 2.09 \times 10^{-3}$ *M*.

[b] 5,5′-Dithiobis(2-nitrobenzoic acid).

[c] 4-Hydroxy-3-nitrophenacyl bromide.

having lesser intensity than that at 279 nm. The ultraviolet and visible spectra show that the chromophoric species present are stable for at least several hours at pH $\leqslant$ 10 at room temperature. It is possible to titrate the 408 nm chromophore spectrophotometrically (Fig. 3), and the measured pK_a of 6.9, in addition to the position of the spectral maximum, suggests the presence of a *p*-nitrophenol moiety on the enzyme. If one

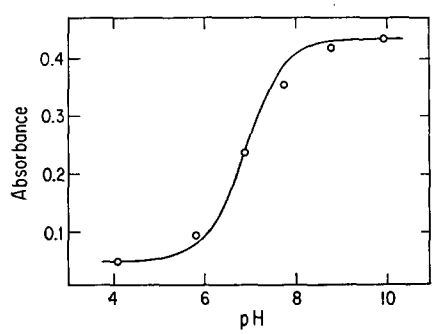


FIG. 3. Titration of the chromophore of modified papain at 408 nm. The solid line is the theoretical curve for $pK_a = 6.9$.

assumes an extinction coefficient of 2×10^4 at 408 nm for the new chromophore, and also assumes that the active enzyme remaining does not contribute to this absorbance, one calculates a ratio of approximately 1.4 moles of modifying moiety per mole of

papain.[3] These results are shown in Table 2. From the constancy of the last column, it can be seen that the attachment of the chromophore to the enzyme parallels the decrease in enzymatic activity.

The modified gel-filtered enzyme was treated with several nucleophiles in an attempt to regenerate active papain or at least to remove the chromophore from the protein. However, as seen in Table 3, treatment of the modified enzyme with sulfhydryl compounds did not remove the modifying group or restore enzymatic activity. Even treatment with hydroxylamine, using the conditions given in Table 3, failed to remove the modifier.

TABLE 2

STOICHIOMETRY OF MODIFIED PAPAIN[a]

Reaction time (sec)	Abs_{278}[b,c]	Abs_{408}[b]	% Activity remaining[d]	Molar ratio modifier/enzyme
120	1.190	0.184	65.3	1.28
300	1.186	0.375	36.7	1.44
450	1.196	0.431	31.5	1.51
1200	1.199	0.400	29.0	1.35
3600	1.183	0.410	29.2	1.41

[a] Modification reaction carried out in 0.05 *M* acetate buffer containing 0.005 *M* EDTA and 1.9% v/v CH_3CN, pH 5.20, $\mu = 0.1$; $[E]_0 = 6.9 \times 10^{-5}$ *M*, $[S]_0 = 2.9 \times 10^{-3}$ *M*.

[b] Measured following a 10:3 dilution by gel filtration.

[c] Corrected for the presence of 18% inactive protein in the starting material.

[d] Measured by assay with Z–GlyPNP.

The nature of the modified enzyme is apparently independent of the pH at which it is prepared, at least in the range pH 5–8. Modified papain prepared at pH 8 not only has spectral properties identical to the material prepared at pH 5.2, when measured under the same conditions, but also fails to react with nucleophiles.

TABLE 3

EFFECT OF ADDED NUCLEOPHILES ON MODIFIED PAPAIN

Nucleophile	pH	Reaction time (hr)	Change in activity[a,b]	% Recovery of absorbance at 408 nm
Cysteine (0.1 *M*)	8.0	16	0.00 ± 0.02	92
$HSCH_2CH_2OH$ (1 *M*)	8.0	4	0.01 ± 0.02	95
NH_2OH (0.5 *M*)	8.5	2	-0.02 ± 0.02	101

[a] This is the fractional change based on hypothetical fully active papain at the given protein concentration.

[b] The activity was determined by assay with Z–GlyPNP.

[3] The deviation of this value from unity may indicate that the extinction coefficient of the modified enzyme is greater than 2×10^4 at 408 nm. Such an increase in the extinction coefficient of an aromatic chromophore near the active site of papain has been observed previously (*15*).

A conventional amino acid analysis of the modified enzyme revealed no obvious changes in amino acid composition relative to the unmodified enzyme. No special precautions were taken to preserve cysteine or tryptophan, so the analysis gives no information about these amino acids; however, the sulfhydryl titration has already identified a cysteine as being involved in the modification of the enzyme. It is worth noting that neither of the two histidines in papain is modified, at least not in a way that would withstand hydrolysis conditions.

Kinetics. In the α-chymotrypsin-catalyzed hydrolysis of I, when $S \gg E$, a rapid increase (burst) in absorbance can be observed, followed by a slower rise which represents steady-state turnover (*4*). To determine if an analogous situation could be seen in the papain case, the enzyme was mixed with I at pH 5.2 in a Durrum–Gibson stopped-flow spectrophotometer and the transmittance monitored at 321 nm. Under conditions where sulfonylation of the enzyme would have produced sufficient nitrophenol to show a transmittance change of *ca.* 10%, assuming a change in extinction coefficient of 4×10^3, as found for the sulfonylation by I of α-chymotrypsin (*17*), such a change was not found. The transmittance observed immediately after mixing was within 1% of the value expected for the starting materials absorbing separately.

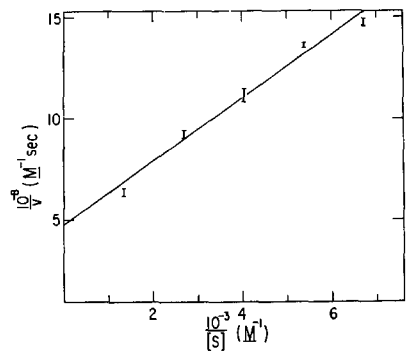


FIG. 4. K_m and k_{cat} are determined by a standard double reciprocal plot using the initial rates of the absorbance increase at 321 nm. The 0.05 *M* acetate buffer contained 0.005 *M* EDTA and 2.3 v/v % CH_3CN, pH 5.20, $\mu = 0.1$. The papain concentration was 8.89×10^{-7} *M*, as determined by Z–Gly PNP assay calibrated by active site titration with 4-hydroxy-3-nitrophenacyl bromide. The slope and intercept, calculated by unweighted least squares treatment of the experimental points, yield $K_m = 3.35 \times 10^{-4}$ *M* and $k_{cat} = 0.240$ sec^{-1}.

Despite the failure of the reaction of I with papain to follow simple Michaelis-Menten kinetics when $S > E$, it is possible to measure the standard kinetic parameters, K_m and k_{cat}, by confining observation to the initial portion of the reaction where inactivation of the enzyme can be neglected. The data of Fig. 4 were obtained during the first 1–1½% of the reaction with appropriate subtractions made for the spontaneous rate at each of the given concentrations.

The value of the ratio K_m/k_{cat} was verified by performing the kinetic determinations with enzyme in large molar excess. Under these conditions, inactivation is not a problem, and pseudo first-order kinetic behavior is observed throughout. A plot of $1/k_{obs}$, where k_{obs} is the observed first-order rate constant for hydrolysis, *vs* $1/[E]$, as given in Fig. 5, shows no measurable deviation from zero at its intercept, but its slope, which is equal to K_m/k_{cat} (*18*), is 1.34×10^{-3} *M* sec., which is consistent with the value of this ratio derived from the experiments with substrate in excess.

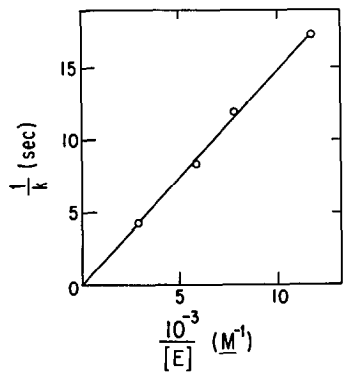
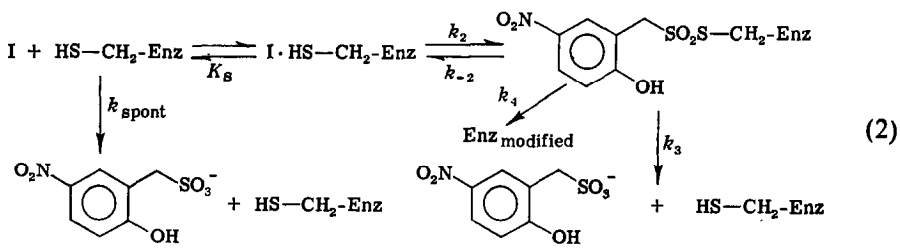


FIG. 5. The value of the ratio K_m/k_{cat} is determined under conditions where the enzyme is in excess. The pseudo first-order rate constant for the absorbance increase at 321 nm is k_{obs} The medium was identical to that described for Fig. 4, with a substrate concentration of 5.0×10^{-6} M. The least squares line yields a value of K_m/k_{cat} of 1.34×10^{-3} M sec.

DISCUSSION

We choose to begin with the assumption that attack of the free sulfhydryl group of papain on the sultone I is an important process. The possibility that the thiolsulfonate so produced is the inactive enzyme observed must be considered. This would be in parallel to the observations of Whitaker (*19*), in which the active site sulfhydryl of papain was reacted with α-toluenesulfonyl fluoride to produce a stable inactive enzyme, presumably the thiolsulfonate derivative. However, this theory requires explaining the enzymatic hydrolysis of the sultone by a different process altogether, in which the thiolsulfonate is not involved. All known examples of such compounds, including the enzymatic derivative α-toluenesulfonyl-papain, decompose readily upon treatment with sulfhydryl compounds, while the present species is very inert.

A more likely explanation is that the thiolsulfonate, once formed, partitions in such a way as to regenerate active enzyme as well as to form an inactive species. This is pictured schematically in Eq. 2.



(2)

It is shown in the Mathematical Appendix that at any point during the course of the reaction, by measuring the rate of disappearance of I and the concentration of the substrate, it is possible to calculate the relative values of k_4, the rate constant for deactivation and k_3, the rate constant for regeneration of active enzyme (turnover). The appropriate relationship is shown in Eq. 3, where k_{cat} and K_m are determined from initial rate measurements under circumstances when the concentration of modified enzyme is

insignificant, S is the spectrophotometrically measured concentration of I at any point during the reaction, v_{enz} is the absolute rate of reaction observed spectrophotometrically, corrected for the rate due to spontaneous hydrolysis at that particular concentration of I, and P_{sp} is the product which has arisen from spontaneous hydrolysis.

$$\frac{k_4}{k_3 + k_4} = \frac{k_{cat}(E_0)(S) - v_{enz}(K_m + S)}{k_{cat}S(S_0 - S - P_{sp})} \tag{3}$$

The constancy of the ratio $k_4/(k_3 + k_4)$ (considering the difficulty in measuring such a small ratio accurately in a single run) throughout the full course of reaction shows that the mechanism of Eq. 2 is consistent with the kinetic behavior observed.[4] This is indicated in Table 4. Furthermore, the data of Table 5 show that this ratio is independent of total initial enzyme and substrate concentrations and thus is a single measurable constant (which may, however, be a function of pH). From these data a value for the ratio $k_3/k_4 = 57$ is obtained. In other words, the thiolsulfonate intermediate of Eq. 2 decomposes to give the inactive modified enzyme less than 2% of the time.

TABLE 4

CALCULATED VALUES OF $k_4/(k_3 + k_4)$ THROUGHOUT THE COMPLETE HYDROLYSIS OF 2-HYDROXY-5-NITRO-α-TOLUENESULFONIC ACID SULTONE[a]

Time (sec)	% (I) reacted[b]	$\frac{k_4}{k_3 + k_4}$
180	8.5	0.020
396	16.5	0.013
696	24.3	0.025
1122	33.1	0.012
2460	54.1	0.030
4026	72.1	0.014
8070	95.7	0.036

[a] The reaction was carried out in 0.05 M acetate buffer containing 0.005 M EDTA and 0.93% v/v CH_3CN, pH 5.20, $\mu = 0.1$; $[E]_0 = 8.05 \times 10^{-7}$ M $[S]_0 = 1.434 \times 10^{-4}$ M. The following kinetic parameters were used: $K_m = 3.349 \times 10^{-4}$ M, $k_{cat} = 0.240$ sec^{-1}, $k_{spont} = 1.194 \times 10^{-4}$ sec^{-1}.

[b] Calculated from the absorbance at 320 nm, using $\epsilon_{(I)} = 2110$, $\epsilon_{(IV)} = 9485$.

With the ratio k_3/k_4 in hand, the entire course of the reaction may be calculated. In Fig. 6 this has been done for a typical run. It may be seen that the correlation of calculated and observed kinetic behavior is excellent. A knowledge of the ratio k_3/k_4 also allows calculation of the rate at which the enzyme is inactivated. When one compares the data of Table 2 with the theoretical values, as is done in Fig. 7, the agreement is also satisfactory.

[4] This, of course, cannot be taken as proof of the mechanism. For example, the scheme given below, corresponding to the mechanism first

$$E_{modified} \xleftarrow{k_4} E\cdot S' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \xrightarrow{k_3} E + P$$

mentioned in this discussion, can be shown to be completely equivalent kinetically. The value measured as the ratio k_3/k_4, following the scheme of Eq. 2 given in the text, would represent $k_1k_3(k_2 + k_4)/k_2k_4(k_1 + k_3)$ according to this alternative.

As yet, we are able only to speculate on the nature of the process by which the thiolsulfonate intermediate of Eq. 2 breaks down. Studies on the organic chemistry, particularly the hydrolytic reactions, of thiolsulfonates appear to be of limited value in

TABLE 5

VALUES OF $k_4/(k_3 + k_4)$ AS A FUNCTION OF VARYING INITIAL ENZYME AND SUBSTRATE CONCENTRATION[a]

10^4 $[S]_0$ (M)	10^6 $[E]_0$[b] (M)	$\frac{k_4}{k_3 + k_4}$[c]
1.134	0.805	0.0172
1.442	0.805	0.0194
1.418	1.073	0.0168
1.418	1.073	0.0177
2.017	1.073	0.0159
2.017	1.073	0.0168
	Average	0.0173 ± 0.0008

[a] All reactions were run under the conditions of footnote *a*, Table 4, except for differences in $[S]_0$ and $[E]_0$.

[b] The concentration was determined by an assay with Z-GlyPNP.

[c] Each value represents a weighted average of 20 calculations made from points at 7–95% of reaction. In practice, the values are calculated essentially from the first half-life, since at later points, v_{enz} is extremely small. Large errors are consequently introduced, and the relative weights decrease.

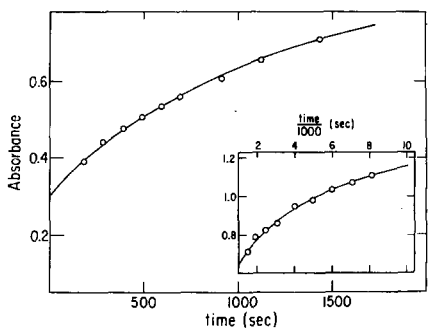


FIG. 6. The kinetics of the enzymatic hydrolysis of I through three half-lives are illustrated. The solid line is calculated using $k_4/(k_3 + k_4) = 0.0173$. Reaction conditions are as noted in Table 4.

understanding the behavior of this enzymatic thiolsulfonate species. In contrast to their oxygen analogs, thiolsulfonates undergo a complex series of reactions during hydrolysis (*20*), which are summarized in Eq. 4.

$$\begin{aligned}
&\text{(a)}\ \mathrm{RSO_2SR' + H_2O} \rightarrow \mathrm{RSO_2H + R'SOH}\\
&\text{(b)}\ \mathrm{R'SOH + RSO_2SR'} \rightarrow \mathrm{RSO_2H + R'SOSR'}\\
&\text{(c)}\ \mathrm{R'SOSR' + H_2O} \rightarrow \mathrm{R'SO_2H + R'SH}\\
&\text{(d)}\ \mathrm{RSO_2SR' + R'SH} \rightarrow \mathrm{RSO_2H + R'SSR'}
\end{aligned} \qquad (4)$$

which has a net overall stoichiometry of:

$$\text{(e)}\ 3\ \mathrm{RSO_2SR' + 2\ H_2O} \rightarrow 3\ \mathrm{RSO_2H + R'SO_2H + R'SSR'}$$

Clearly, this set of reactions cannot apply to the enzymatic case. (Step b would represent a dimerization of enzyme molecules!) The primary problem is that a thiolsulfonate normally suffers nucleophilic attack at the sulfenyl sulfur, eliminating sulfinate, and generating the powerfully nucleophilic (*21*) sulfenic acid (Eq. 4a). While the latter species can react with a molecule of thiolsulfonate in free solution, this seems most unlikely when the thiolsulfonate is another enzyme molecule.[5]

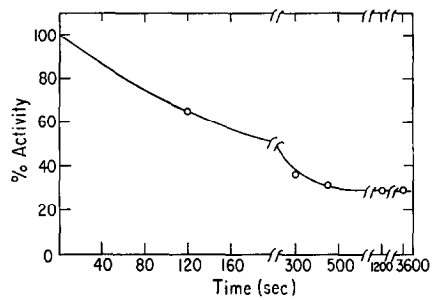
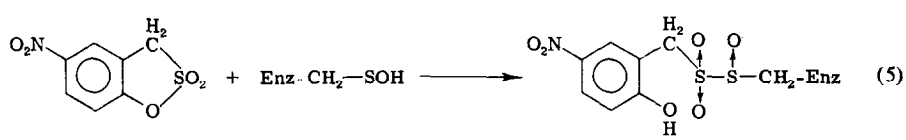


FIG. 7. The inactivation of papain by I is shown. The solid line represents enzyme activity calculated using $k_4/(k_3 + k_4) = 0.0173$. The circles represent the determinations of Table 2.

In fact, barring any special reductions that would occur later, it appears that the enzymatic thiolsulfonate species must decompose *without nucleophilic substitution at the sulfenyl sulfur*. Except for the high temperature elimination of SO_2 (*23*), this is, as far as we are aware, the first observation of such a reaction. The closest parallel would seem to be the hydrolysis of Bunte salts, $RSSO_3^-$, which undergo only electrophilic catalysis at the sulfenyl sulfur and cleave to generate the corresponding thiol (*24*).

While the reason for the special reactivity[6] of this thiolsulfonate species is not yet clear, it must be due to the presence of the phenolic hydroxyl, since the similar thiolsulfonate generated by reaction of papain with α-toluenesulfonyl fluoride is completely stable under our conditions (*19*). This difference in behavior between the 2-hydroxy-5-nitro-α-toluenesulfonyl-enzyme and the α-toluenesulfonyl species is analogous to the difference between the corresponding species in the α-chymotrypsin case which was mentioned in the Introduction to this paper. For the latter enzyme, the possibility that the phenolic hydroxyl group acts as a general acid catalyst has been suggested (*1*). A similar explanation may be valid for the papain case.[7]

[5] The reaction of the sulfenic acid with another molecule of the sultone, as shown in scheme 5, would appear to be more likely. The sulfinyl sulfone thus formed, however, would be expected to decompose to two molecules of sulfinate, which would not account for the enzymatic reaction (*22*).



(5)

[6] This reactivity is even more surprising considering the fact that despite the excellence of *p*-nitrophenols as leaving groups, the *formation* of thiolsulfonate is apparently rate-determining under all of the conditions we employed in studying the reaction of I with papain.

[7] In intramolecular cases, general acid catalysis has been observed even in the hydrolysis of esters of strong acids (*25*).

The nature of the secondary reaction pathway, resulting in inactive enzyme, is also uncertain; indeed, the product is not fully characterized. We know only that the modified enzyme contains a *p*-nitrophenol chromophore and that this chromophore is bound to the enzyme by a linkage which resists rather vigorous treatment with nucleophiles. It does not seem, however, that residues other than cysteine are altered in the modification of the enzyme. One possibility which is being considered is that an internal rearrangement of the thiolsulfonate to give a very stable enzyme-bound species takes place. Another is that the thiolsulfonate (perhaps with acid catalysis) undergoes carbon–sulfur bond cleavage. This process would generate a thiolsulfonate anion with the concomitant formation of the hydroxy analog of papain, which then might be sulfonylated by I, this time irreversibly. Such carbon–sulfur bond cleavage, with the development of some carbonium ion character at the carbon, has been observed, albeit under rather different conditions (*23*). It should be noted that the carbon–sulfur bond cleavage reaction would only have to occur 2% of the time in the breakdown of 2-hydroxy-5-nitro-α-toluenesulfonyl-papain to account for our data.

We are presently pursuing the investigation of the nature of the inactivated enzyme.

EXPERIMENTAL SECTION

All spectrophotometric measurements were made on a Cary 15 or a Gilford Model 222 spectrophotometer. pH measurements were made on a Radiometer type PHM4c pH meter equipped with an Arthur H. Thomas model 4858-L15 combination glass-calomel electrode. Calibrations were made against Fisher standard buffers.

Materials. Papain, purchased from Worthington Biochemical Corporation, was purified by affinity chromatography following the method of Blumberg *et al.* (*26*). Batches of enzyme so prepared had purities ranging from 75–90%, as estimated by active site titration (*15*). In general, the enzyme was mercurated after purification and reactivated immediately before use by gently shaking with 1000-fold excess of *p*-toluenethiol in an equal volume of toluene, followed by gel filtration on Sephadex G-25. Mercurated papain stored at 2°C showed no loss of activity over several weeks.

2-Hydroxy-5-nitro-α-toluenesulfonic acid sultone, prepared by direct nitration of the parent sultone (*27*), was a gift from Mr. William Berg. 4-Hydroxy-3-nitrophenacyl bromide (*28*) was a gift from Mr. Richard W. Furlanetto. Hydroxylamine hydrochloride, purchased from Matheson, Coleman and Bell, was recrystallized from methanol and dried *in vacuo* over calcium sulfate immediately before use. All other materials employed were from commercial sources and used without further purification.

Amino acid analyses were performed on a Beckman amino acid analyzer with electronic integration. The modified enzyme was prepared for analysis as follows. To 70 ml of a 10^{-6} *M* solution of papain in 0.05 *M* acetate buffer, $\mu = 0.1$, pH 5.2, containing 0.005 *M* EDTA, was added 0.015 g of I dissolved in 1 ml of acetonitrile. After several hours at room temperature, the enzyme solution was reduced in volume to about 4 ml by filtration through a UM10 Diaflow Ultrafilter mounted in an Amicon model 8 MC Microultrafiltration system. The concentrated sample was filtered through Sephadex G-25 with distilled water as the eluant, lyophilized, and dissolved in 5 ml of constant boiling HCl. After freeze–thaw degassing at a pressure of 0.005 mm Hg, the glass container holding the sample was sealed and heated at 110°C in an oil bath for 24 hr. The azeotropic solvent was then removed under vacuum.

Kinetic measurements. In the usual experiments, the buffered enzyme solution was equilibrated in a quartz UV cell of 1-cm pathlength in the thermostatted cell compartment of the spectrophotometer employed. A measured volume of a solution of I in acetonitrile was introduced on a flat-tipped stirring rod. The concentration of I was monitored by observing the appearance of the product at 321 nm.

The nonenzymatic hydrolysis of I was measured spectrophotometrically at 321 nm. The reaction was clearly first-order through at least three half-lives and the observed rate constant at pH 5.2 in acetate buffer was $(1.20 \pm 0.05) \times 10^{-4}$ sec^{-1}. Extrapolating the absorbance readings in this reaction to the time of mixing and noting the infinity value (8–10 half-lives), afforded values for the extinction coefficients for I and IV at 321 nm of 2110 and 9485, respectively.

TABLE 6

EFFECT OF PRODUCT SULFONIC ACID ON PAPAIN ACTIVITY AT 25.0°

Substrate	Sulfonic acid concentration		
	0	0.615×10^{-4} *M*	1.23×10^{-4} *M*
I[a]	5.98[c]	5.83[c]	5.93[c]
Z–Gly PNP[b]	1[d]	1.01[d]	1.00[d]

[a] Assay conditions were pH 5.20, 0.05 *M* acetate buffer, containing 0.005 *M* EDTA and 1.9% v/v CH_3CN, with $\mu = 0.1$; $[E]_0 = 1.40 \times 10^{-6}$ *M*, $[S]_0 = 9.68 \times 10^{-5}$ *M*.

[b] Assay conditions were pH 6.80, 0.02 *M* phosphate buffer, containing 0.001 *M* EDTA and 0.93% v/v CH_3CN; $[E]_0 = 1.82 \times 10^{-7}$ *M*, $[S]_0 = 1.20 \times 10^{-4}$ *M*.

[c] Initial rate $\times 10^8$, in units of *M* sec^{-1}, measured at 321 nm, and using $\Delta\epsilon = 7375$.

[d] Zero order rate relative to that with the concentration of [IV] = 0, measured at 400 nm.

Product inhibition. Solutions of IV of concentrations 1.23×10^{-4} *M* and 0.615×10^{-4} *M* were prepared by allowing I to be hydrolyzed either at pH 5.2 (acetate buffer) or pH 6.8 (phosphate buffer) in the absence of enzyme. Freshly activated papain was then added to the solutions of IV and incubated for up to 30 min. The resultant solutions were assayed for activity toward I or in the standard assay with Z–GlyPNP. The results, indicating the complete absence of product inhibition, are given in Table 6.

Papain inactivation. After addition of I to the enzyme solution, the ensuing reaction was effectively quenched after the desired time by gel filtration on a Sephadex G-25 column. The error in determining the reaction time is on the order of 10% of the time for elution of the null volume, or about 50 sec. The inactivation results of Tables 1 and 2 reflect the activity relative to control samples of papain treated identically, except for the addition of I. A typical control sample shows a small activity loss (<10%) over the period of the experiment. Absorbance measurements at 279 nm showed that typically 94–97% of the protein was recovered after treatment by gel filtration.

ACKNOWLEDGMENTS

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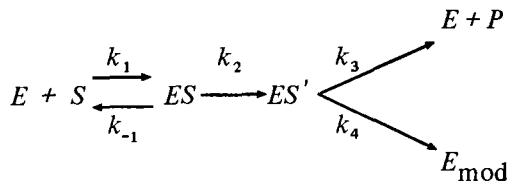
APPENDIX

If the mechanism of Eq. 1 is assumed, then from the steady-state and material balance considerations equations (A2), (A3) and (A4) can be written



(A1)

$$\frac{d(ES)}{dt} = 0 = k_1(E)(S) - (k_{-1} + k_2)(ES) \tag{A2}$$

$$\frac{d(ES')}{dt} = 0 = k_2(ES) - (k_3 + k_4)(ES') \tag{A3}$$

$$E_0 = E + ES + ES' + E_{\text{mod}} \tag{A4}$$

Solving for ES' gives Eq. (A5).

$$ES' = \frac{k_1(E_0 - E_{\text{mod}})S}{\dfrac{k_1(k_2 + k_3 + k_4)}{k_2}S + \dfrac{(k_{-1} + k_2)(k_3 + k_4)}{k_2}} \tag{A5}$$

The enzymatic rate, $v_{\text{enz}} = v_{\text{obs}} - v_{\text{spont}}$, is given by Eq. (A6) where $K_s = (k_{-1} + k_2)/k_1$,

$$\begin{aligned} v_{\text{enz}} = (k_3 + k_4)(ES') &= \frac{k_2(k_3 + k_4)(E_0 - E_{\text{mod}})S}{(k_2 + k_3 + k_4)S + \dfrac{k_{-1} + k_2}{k_1}(k_3 + k_4)} \\ &= \frac{\dfrac{k_2(k_3 + k_4)}{(k_2 + k_3 + k_4)}(E_0 - E_{\text{mod}})S}{S + K_s\dfrac{k_3 + k_4}{k_2 + k_3 + k_4}} \end{aligned} \tag{A6}$$

From initial rate measurements, where $E_0 \gg E_{\text{mod}}$, we measure a

$$k_{\text{cat}} = \frac{k_2(k_3 + k_4)}{k_2 + k_3 + k_4}$$

and a

$$K_m = K_s\frac{k_3 + k_4}{k_2 + k_3 + k_4}.$$

Thus over the total course of the reaction we have Eq. (A7).

$$v_{\text{enz}} = \frac{k_{\text{cat}}(E_0 - E_{\text{mod}})S}{S + K_m} \tag{A7}$$

However, E_{mod} is simply equal to the fraction of substrate having reacted by path k_4, as shown by Eq. (A8), where P_{sp} is the concentration of product formed by spontaneous hydrolysis, determined by numerical evaluation of the integral of Eq. (A9)

$$E_{\text{mod}} = \frac{k_4}{k_3 + k_4}(S_0 - S - P_{\text{sp}}) \tag{A8}$$

$$P_{\text{sp}} = \int_0^t k_{\text{sp}}\,S(t)\,dt \tag{A9}$$

Substituting Eq. (A8) in Eq. (A7) gives Eq. (10) which can be rearranged to Eq. (A11), corresponding to Eq. (3) in the text.

$$v_{enz} = \frac{k_{cat}\left[E_0 - \frac{k_4}{k_3 + k_4}(S_0 - S - P_{sp})\right]S}{S + K_m} \tag{A10}$$

$$\frac{k_4}{k_3 + k_4} = \frac{k_{cat}(E_0)(S) - v_{enz}(K_m + S)}{k_{cat}S(S_0 - S - P_{sp})} \tag{A11}$$

In practice, both rates and concentrations are measured spectrophotometrically, and the assumption is made that both products, IV and the modified enzyme, have equal extinction coefficients. An error in this assumption will be reflected directly in the ratio $k_3/(k_3 + k_4)$. In a model case, the sulfonyl enzyme formed by the acylation of α-chymotrypsin by I has an extinction coefficient of 6825(5) at 320 nm, compared to 9490 for the free sulfonate anion IV.

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