THE INTRAMOLECULAR NUCLEOPHILIC REACTION OF A PHENOLIC HYDROXYL GROUP AT THE ACTIVE SITE OF PAPAIN

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Received July 10,1972

Summary: Sulfonylation of the sulfhydryl protease papain with β-(2-hydroxy-3,5-dinitrophenyl)ethanesulfonic acid sultone 1 at pH 5.2 and 25° gives a thiolsulfonate species 2, the desulfonylation of which has been studied. Recyclization to give the sultone 1 *via* the nucleophilic attack of the phenolic hydroxyl group in 2 on the sulfonyl function has been found to compete effectively with hydrolysis. This is the first case in which a recyclization reaction has been observed where the central atom of the newly generated nucleophilic group produced by the ring-opening reaction of a cyclic ester with a protease is different in kind from that present in the active site of the enzyme.

Recently, it has been demonstrated that phenolic hydroxyl groups produced by the ring-opening reactions of aromatic lactones, sultones and cyclic phosphoric acid esters with the active site serine hydroxyl in α-chymotrypsin can act as effective intramolecular nucleophiles, attacking the corresponding acyl, sulfonyl, or phosphoryl functions and regenerating the cyclic esters and active enzyme (1). The steric proximity of the phenolic hydroxyl groups to the latter functions allows the thermodynamically unfavorable recyclization reactions to occur at rates which compete favorably with hydrolysis.

We wish now to report the first case in which a recyclization reaction has been observed where the central atom of the newly generated nucleophilic group

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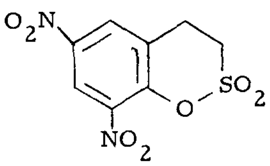
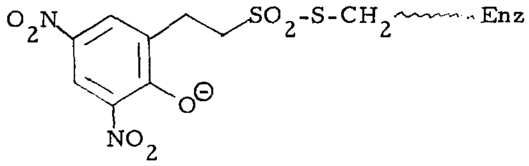
is different in kind from that present in the active site nucleophile of the enzyme. When the sulfhydryl protease papain (2) is reacted with β-(2-hydroxy-3,5-dinitrophenyl)ethanesulfonic acid sultone (3) 1 in acetate buffer at pH 5.2, the enzyme is cleanly and rapidly sulfonylated. Complete loss of activity and sulfhydryl content occurs and a new chromophore (λ_{max} = 373 nm, 405 nm), indicative of a dinitrophenoxide moiety, is produced. Removal of excess sultone by gel filtration allows observation of the behavior of the sulfonyl-enzyme 2.



1



2

Enzymatic activity, measured by hydrolytic activity toward p-nitrophenyl N-(benzyloxycarbonyl)glycinate, (4) is restored upon standing in acetate buffer. A maximum activity of 8.6 ± 1.9% of the activity of the starting enzyme was restored after 18 hrs, at which time a slow and nearly linear decline in activity was observed (Figure 1).

The increase in enzymatic activity is paralleled by a decrease in the solution's absorbance at 400 nm. After 38 hr, the value reached a minimum, which represents a disappearance of 11.3 ± 1.0% of the original chromophore. The observed kinetics for both the reactivation process and the absorbance change lie between first and second order; measurements for the first half-life are 4.5 ± 0.3 hr (absorbance) and 4 ± 1 hr (reactivation).

When mercuric chloride or sodium chloroacetate, both of which react rapidly and quantitatively with the active enzyme (2) are added to the sulfonyl-enzyme solution, considerably different behavior is observed. Although reactivation rates cannot be studied, the absorbance decrease at 400 nm is cleanly first-

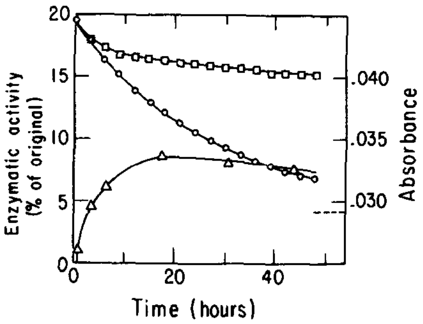


Figure 1. --The reactions of $\underline{2}$ in 0.05 $\underline{M}$ acetate buffer, pH 5.20, at 25°C.: △-enzymatic activity as measured by standard assay; □-absorbance change at 400 nm; O-absorbance change when 0.028 $\underline{M}$ sodium chloroacetate is present, 0.003 $\underline{M}$ $HgCl_2$ produces an identical curve. The dashed line represents the calculated infinity absorbance of the chloroacetate reaction.

order and proceeds to give a much smaller absorbance value, representing a loss of 38.2 ± 1.2% of the chromophore (Figure 1). Both the rates and final absorbance values are, within experimental error, independent of the choice of inactivating agent and of its concentration (always in excess over the enzyme).

Under these conditions of enzyme scavenging, the presence of $\underline{1}$ in the product mixture can be demonstrated.* Ether extraction and subsequent thin layer chromatography of the organic phase on silica gel plates shows a single spot in two solvents. The R_f values, 0.2 in benzene, 0.45 in chloroform, are identical to those observed for an authentic sample of $\underline{1}$. In contrast, a control extraction performed on a freshly-generated, gel-filtered solution of $\underline{2}$ showed no detectable sultone as a contaminant. These data, particularly the isolation of $\underline{1}$ as a reaction product, are explained by a set of reactions (Scheme 1) in-

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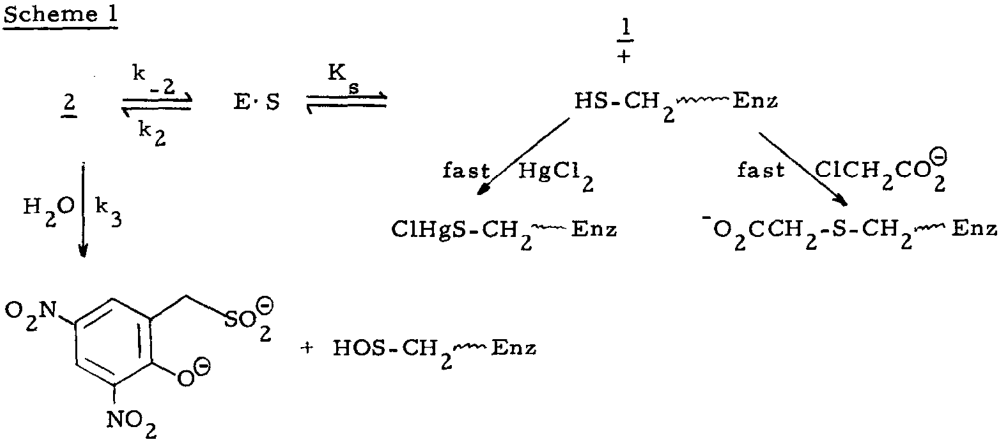
*The hydrolysis of $\underline{1}$ in the absence of enzyme is immeasurably slow under these conditions.

volving recyclization of the sulfonyl-enzyme (k_{-2}), resulting from an attack of the phenolic hydroxyl on the sulfonyl sulfur. Also involved is a competing reaction, here represented by k_3 and shown as the first step in normal (1c) thiolsulfonate hydrolysis.* The product sulfinate retains the dinitrophenoxide moiety and would be expected to absorb nearly equally to 2 at 400 nm. The absorbance decrease observed is then due to step k_{-2} and is paralleled by production of the active enzyme. The k_{-2}/k_2 equilibrium lies to the left under these conditions, but is shifted as mercuric chloride or chloroacetate irreversibly drains off the free enzyme.

Scheme 1



Two interesting predictions arise from this scheme. First, the dinitrophenoxide chromophore which remains after several days should be no longer covalently bound to the enzyme, and second, the enzyme activity should be re-

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*The enzyme product is shown here as a sulfenic acid. Although only three sulfenic acids are known, evidence has recently accumulated that such species may be stable at the active site of sulfhydryl enzymes (5).

storable by treatment with sulfhydryl compounds,* except for that fraction corresponding to irreversible alkylation by chloroacetate.

Both predictions have been confirmed. Gel filtration of the product mixture after three days (four half-lives) produces a protein fraction having an absorbance at 400 nm of only 5 ± 2% (calculated = 6%) of the original value. Moreover, treatment of the final product mixtures with p-toluenethiol regenerates nearly complete activity, except when chloroacetate has been used as an enzyme scavenger.

The rate constants in Scheme 1 can be estimated. The sum of k_{-2} and k_3 is simply the observed first-order rate constant for the absorbance change in the presence of enzyme inhibitor. Their ratio is calculated from the final absorbance under the assumption that the k_3 step gives no absorbance change. This treatment yields values of $4.0 \pm 0.5 \times 10^{-6}$ $\sec^{-1}$ and $6.2 \pm 0.5 \times 10^{-6}$ $\sec^{-1}$ for k_{-2} and k_3, respectively at 25.0°. Independent measurement of the rate of the sulfonylation reactions at 25.0° shows $k_2/K_s = 91 \pm 4$ $\underline{M}^{-1}$ $\sec^{-1}$ with $K_s \geq 10^{-3}$ $\underline{M}$.

In principle, the knowledge of these rate constants allows calculation of the predicted behavior of 2 in the absence of enzyme inhibitors. However, since in the time course of these experiments (several days), enzyme inactivation from other causes, denaturation, oxidation, autolysis, etc., becomes a serious problem, such an analysis has not been attempted. Order of magnitude estimates indicate that the behavior in the first few hours is consistent with our proposed scheme.

It is interesting to note the efficiency of an oxygen nucleophile operating at

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*The reactivation of mercurated papain by thiols is well known (2). Sulfenic acids react with thiols to produce disulfides (6a), which then may be further reduced. Similarly, likely products from a sulfenic acid intermediate also would be eventually reduced to thiols (6b).

the active site of an enzyme whose normal mechanism entails the use of a sulfur nucleophile. Recyclization to a six-membered ester is expected to be, by several orders of magnitude, kinetically less favored, relative to attack by solvent, than that to a five-membered ester (1b).[*,**] Nevertheless, we observe such closure as one of the two predominant pathways for reaction of the sulfonyl-enzyme 2.

A related comparison of oxygen and sulfur analogs is provided by the case of subtilisin and thiosubtilisin (7) where substitution of sulfur for oxygen seriously impairs the efficiency of enzymatic catalysis (8). Our example shows that in at least one transformation of the reverse type, the foreign nucleophile may be very efficient indeed.

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[*] Recyclization to the homologous five-membered sultone cannot be studied in the papain system because of competing reactions (1c).

[**] We have found that the thiolsulfonate species analogous to 2 formed by the reaction of 1 with N-acetylcysteine is completely stable for several days under the reaction conditions employed in the papain studies.

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