Studies on the Desulfonylation of 2-Hydroxy-3,5-dinitro-α-toluenesulfonyl-α-chymotrypsin

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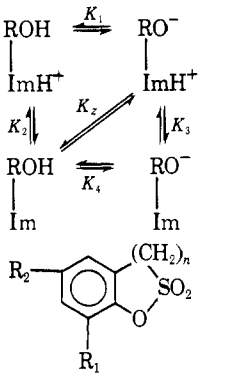
Abstract: Reaction of 2-hydroxy-3,5-dinitro-α-toluenesulfonic acid sultone with α-chymotrypsin produces a sulfonyl enzyme containing a phenolic hydroxyl covalently attached at the active site. Rate constants for the slow desulfonylation of 2-hydroxy-3,5-dinitro-α-toluenesulfonyl-α-chymotrypsin have been obtained from a study of the initial rate of recovery of activity toward the specific substrate *N*-acetyl-L-tryptophan methyl ester. In conjunction with the findings of an earlier study of similar desulfonylation reactions, the pH dependence and maximum value of the rate constants reveal that the un-ionized form of the phenolic hydroxyl group and un-ionized form of the active site residue histidine-57 are required for the decomposition of such sulfonyl enzymes. Because the phenol residue can act as an intramolecular nucleophile, this demonstrates that these desulfonylation reactions have the same mechanistic requirements as chymotrypsin deacylation reactions with specific carboxylic acid substrates: dependency on imidazole as a base and dependency on the reacting nucleophile as an un-ionized acid.

α-Chymotrypsin (Ct) reacts rapidly and stoichiometrically with the cyclic sulfonic acid ester, 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I), to form a catalytically inactive enzyme, I_{Ct}, sulfonylated on the active site serine hydroxyl.[1] Unlike unsubstituted aryl- and alkylsulfonylchymotrypsins,[2] this derivative desulfonylates near neutral pH, exhibiting a bell-shaped pH-rate profile.[1,3] This pH dependence has been interpreted in terms of the scheme in eq 1, where ROH represents the phenol and Im, the histidine-57 imidazole in the sulfonyl enzyme. However, the scheme is ambiguous because the reaction of either of the formally neutral species, Im-ROH or ImH^+-RO^-, would produce the same pH dependence. That is, the ratio of the concentrations of the two formally neutral forms is independent of the pH, and therefore the pH dependence of the rate constant for desulfonylation cannot unambiguously identify the species which desulfonylates. Because most sulfonic acid derivatives of Ct are stable, one cannot argue with confidence by analogy with the reactions of rapidly enzymatically hydrolyzed substrates derived from carboxylic acids that the imidazole of histidine-57 must be unprotonated, allowing it to act as general base catalyst for desulfonylation. Rather, an identification of the reactive species in eq 1 should reveal whether or not such desulfonylation reactions are mechanistically similar to the deacylation reactions of normal ester and amide substrates of Ct.

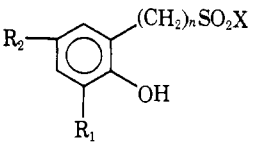
The similar values of pK_1 and pK_2, calculated from the measured rate constants for the desulfonylation of I_{Ct}[3] according to eq 1, indicated that the two formally neutral species Im-ROH and ImH^+-RO^- exist in nearly equal amounts in this case. This led to our decision to try to identify the reactive species in the desulfonylation process by determining the relationship of changes in the rate of desulfonylation to alterations in the composition of the equilibrium mixture of the formally neutral species Im-ROH and ImH^+-RO^-. Any modification reducing the composition of this equilibrium mixture to a single major species without greatly altering the structure of the sulfonyl enzyme should permit the identification of the reactive species by the observation of the concomitant increase or decrease in rate proportional to the change in concentration of that species.

Preliminary studies in this laboratory indicated that 2-hydroxy-3,5-dinitro-α-toluenesulfonyl-α-chymotrypsin (II_{Ct}) which contains a more acidic *o*-hydroxyl group than I_{Ct} does is considerably more stable than the latter species.[1] Thus, the presence of an additional nitro group which should cause the predominant form of the formally neutral



(1)

n	R_1	R_2	compd no.
1	H	NO_2	I
1	NO_2	NO_2	II
2	NO_2	NO_2	III



n	R_1	R_2	X	compd no.
1	H	NO_2	OCt	I_{Ct}
1	NO_2	NO_2	OCt	II_{Ct}
2	NO_2	NO_2	OCt	III_{Ct}
1	NO_2	NO_2	O^-	II_a
2	NO_2	NO_2	O^-	III_a
1	H	NO_2	OCH_3	I_{me}
1	NO_2	NO_2	OCH_3	II_{me}
2	NO_2	NO_2	OCH_3	III_{me}

species to be the zwitterion ImH^+-RO^- results in the stabilization of the sulfonyl derivative. This fragmentary evidence does not constitute proof that Im-ROH is the reactive species in the desulfonylation process because most sulfonyl enzymes are stable. The purpose of the present paper is to report a kinetic study which directly correlates a reduction in the rate of desulfonylation of II_{Ct} relative to I_{Ct} with a corresponding reduction in the concentration of Im-ROH relative to that of ImH^+-RO^-. We obtained the rate constant for the slow enzymatic desulfonylation of II_{Ct} from a study of the initial rate of the resultant recovery of enzymatic activity toward the specific substrate *N*-acetyl-L-tryptophan methyl ester. The "reporter" properties of the covalently attached nitrophenol group in II_{Ct} provided in-

Table I. Properties of Methyl Sulfonates

Methyl sulfonate	Visible spectra[a] λ_{max}, nm	ϵ_{max}, M^{-1} cm^{-1}	Mp, °C	pK_a
I_{me}	400	22,000	157	6.51
II_{me}	365	16,000	155.5	2.90
III_{me}	368	15,500	112.8–113.2	3.93

[a] Nitrophenoxide absorbance in water.

formation about the composition of the equilibrium mixture containing Im-ROH and ImH^+-RO^- and about the environment of the reacting moieties.

Experimental Section

Materials. α-Chymotrypsin was obtained from the Worthington Biochemical Corp. (lot CDI 11B). Solutions of enzyme were prepared by dissolving a given weight of dry enzyme in the appropriate buffer immediately before use. Titration with *N-trans*-cinnamoylimidazole[4] indicated an active site concentration corresponding to 84.3% of the protein concentration.

2-Hydroxy-3,5-dinitro-α-toluenesulfonic acid sultone (II)[5] and β-(2-hydroxy-3,5-dinitrophenyl)ethanesulfonic acid sultone (III)[6] were gifts from Dr. K.-W. Lo and were recrystallized from dry ethanol before use. *N*-Acetyl-L-tryptophan methyl ester (ATME), a gift from Dr. J. Heidema,[3] was recrystallized twice from $CHCl_3$-cyclohexane. Solutions of the sultones and ATME were prepared in acetonitrile (J. T. Baker Chemical Co.) which was distilled from P_2O_5 within 24 hr of its use. All inorganic compounds employed in the preparation of buffer solutions were of reagent grade. Fisher Certified buffers were used as standards in all pH determinations. All water used in this work was distilled and then passed through a mixed-bed ion exchange column (Continental Demineralization Service).

Preparation and Spectral Characteristics of Sulfonyl-α-chymotrypsins. In 5 ml of 5×10^{-2} *M* phosphate buffer, pH 7.5, was dissolved 1.5×10^{-2} g of Ct. To this solution at room temperature 0.2 ml of a 5×10^{-2} *M* solution of the sultone (II or III) in dry CH_3CN was slowly added with stirring. After the development of yellow color ceased, the solution was gel-filtered on Sephadex G-25 with 10^{-3} *M* acetate buffer, pH 5, at 0°. The first yellow band (immediately after the void volume) was collected and made 1 *N* in NaCl. Repeated gel filtration of this fraction revealed a constant ratio between the protein absorbance at 280 nm and the dinitrophenoxide absorbance at 367 nm. From the stock solution obtained, 1-ml aliquots were added to 1 ml of buffers which were at 0.05 ionic strength, and the absorbances of the resultant solutions were recorded from 350 to 700 nm. In the lower pH range precipitation problems were encountered necessitating rapid mixing of solutions and extrapolation of the absorbances measured at a single wavelength to time zero.

Methyl Sulfonates I_{me}, II_{me}, and III_{me} Derived from the Corresponding Sultones. In 2 ml of dry, freshly distilled dioxane 0.2 g of the appropriate sultone was dissolved. The addition of 2 ml of a freshly prepared, clear solution of 60 mg of sodium methoxide in methanol resulted in a rapid development of yellow color. Concentrated HCl was added until the solution was colorless, and the solution was taken to dryness with a rotary evaporator. Extraction of the residue with chloroform and subsequent evaporation of the solvent yielded oils which crystallized after 24 hr. Purification of the methyl esters included fractional crystallization from Et_2O-petroleum ether and recrystallization from Et_2O. Solutions of these compounds in dilute NaOH acquire the yellow color characteristic of nitrophenols. Satisfactory ir, uv-visible, NMR, and mass spectra and thin-layer chromatograms were obtained for all the compounds. The visible spectra of the nitrophenol groups were measured at various pH values with the same procedure as was used for the enzyme derivatives. The pK_a values were determined graphically and by a computer analysis from the sigmoidal changes in absorbances vs. pH that were seen. These pK_a values are listed in Table I together with appropriate spectral data and the melting point for each compound.

General Experimental Techniques. All glassware was routinely washed with chromic acid and rinsed several times with distilled deionized water. A Cary 15 recording spectrophotometer equipped with a thermostatable cell compartment and cell jacket was employed for uv-visible spectral measurements. A Lauda K2 water bath was used to maintain a constant temperature for the kinetic studies. The reactions were carried out in 1-cm path length quartz cuvettes. Measurements of pH were made either on a Radiometer Model pH M 4C or a Beckman Research pH meter. Temperature readings were made with thermometers calibrated against a National Bureau of Standards Thermometer.

Kinetics of the Reaction of Ct and Sultone III. The kinetics of the sulfonylation of Ct by III were determined with the enzyme in excess. From a stock solution containing 10^{-3} *M* Ct, 0.2 ml was added to 2.0 ml of the appropriate 0.05 *M* buffer (pH range, pH 5.6–8.87). The reaction was initiated by the addition of 0.01 ml of 1.93×10^{-3} *M* III in dry acetonitrile and was followed at 360 nm. Pseudo-first-order rate constants were determined from the slopes of the linear plots of log $(A_\infty - A_t)$ vs. time. At higher pH values it was necessary to determine the rate of the spontaneous hydrolysis of III and the rate constants obtained for the sulfonylation of the enzyme were corrected for the spontaneous process.

Reactivation of Sulfonylchymotrypsins II_{Ct} and III_{Ct}. The initial rate of reactivation of II_{Ct} was determined using only solutions of enzyme which were at least 98% inactivated. We dissolved 9×10^{-3} g of Ct in 30 ml of 0.01 *M* phosphate buffer, pH 7.3, and assayed an aliquot of the solution with ATME. To 28 ml of the solution we added 0.05 ml of 1.12×10^{-2} *M* II in CH_3CN over 60 sec at 25° with stirring. We diluted aliquots of this solution with equal volumes of 1.0 ionic strength buffers (NaCl). We thermostated the resulting solutions at 25° and determined their activity at various times by rate assay with ATME. The first-order rate constants for recovery of enzymatic activity were calculated as described in the Results section.

In a typical experiment with III_{Ct}, 0.2 ml of the stock solution of this species was added to 5.0 ml (final volume) of 0.525 ionic strength buffers at pH values of 4, 5, and 7. The resultant samples were assayed daily by the following procedure to determine the concentration of active Ct. A solution of 0.05 ml of 4×10^{-2} *M* ATME in acetonitrile was added to 2.0 ml of 10^{-3} *M* phosphate buffer, pH 6.8, which was temperature-equilibrated in the thermostated cell compartment of a Cary 15 spectrophotometer for about 15 min. The solution to be assayed (0.1 ml) was then added. The reaction velocity for the hydrolysis of ATME determined from the slope of the initial, linear portion of the absorbance vs. time curve is used here as a measure of the concentration of active enzyme. In control experiments pure acetonitrile was used in place of the sultone solutions in the preparation of the enzyme stock solutions.

Results

Preparation of Sulfonyl Enzymes. When a small excess of sultone II is efficiently mixed with a 10^{-5} *M* solution of Ct at pH 7.3 and 25°, a burst in absorbance with λ_{max} 371 nm (ϵ 13,900 M^{-1} cm^{-1}, gel-filtered product) is observed, with a concomitant loss of at least 98% of the enzyme's catalytic activity toward ATME. Gel filtration on Sephadex G-25 indicates that the chromophore is covalently attached to the protein. The extremely rapid hydrolysis of II at neutral pH made it impractical to study the kinetics of this reaction.

The six-membered dinitro-substituted sultone III also reacts readily with Ct producing a stable intermediate with concomitant formation of a dinitrophenoxide chromophore. Gel filtration on Sephadex G-25 at pH 5 and the loss of catalytic activity of the gel-filtered species toward ATME reveal that this chromophore is also covalently attached to the inhibited enzyme.

We investigated the kinetics of the sulfonylation of Ct by III to verify that the reaction is similar to the reactions of the five-membered sultones with this enzyme. Although we used an excess of enzyme with respect to substrate, above pH 8 it was necessary to correct the observed rate constant for the nonenzymatic hydrolysis of III. With a sufficient excess of Ct, semilogarithmic plots of the concentration of unreacted sultone vs. time indicate that the reaction is pseudo-first-order during its entire course. Values of the pseudo-

first-order rate constants (k) were obtained from the slopes of these plots. Under conditions of $E > S$ and sufficiently low enzyme concentration, $k/E = k_2/K_s$. As seen in Figure 1 the pH dependency of k_2/K_s is bell shaped and suggests that the rate of sulfonylation of Ct by III depends on ionizing groups in the enzyme with pK_a values of 7.01 and 8.48, respectively.[1] The value of $(k_2/K_s)_{lim}$ calculated is 11.3 M^{-1} sec^{-1}. Because the pH dependency of k_2/K_s for this sulfonylation reaction is similar to that for a large variety of reactions in which the hydroxyl group of serine-195 is the reactive group, we assume that the sulfonylated species formed is also a serine ester.

Reporter Properties of Nitrophenols. Both of the sulfonyl enzymes, II_{Ct} and III_{Ct}, are sufficiently stable that the spectra of the covalently bound nitrophenol chromophores present in these species can be obtained over a wide pH range after gel filtration. Several of these spectra are compared in Figure 2 with those for a related sulfonic acid, III_a.

The absence of any isosbestic points in the spectra of III_{Ct} from 350 to 500 nm as pH is varied suggests that only a single absorbing species is being detected with λ_{max} 368 nm (ϵ 13,300 M^{-1} cm^{-1}). The shape of these spectra seen, also, in the corresponding spectra of compound III_a is characteristic of 2,4-dinitrophenoxide-2,4-dinitrophenol systems. A plot of the absorbance at 360 nm vs. pH fits a theoretical sigmoid based on a pK_a value of 4.69. The slight increase (0.8 units) in this value over the pK_a of the analogous methyl sulfonate, III_{me} (see Table I), suggests that the ionization of the enzyme-bound dinitrophenol group might occur in a somewhat hydrophobic environment.

The spectral properties of II_{Ct} are quite different. In the lower pH region it behaves similarly to III_{Ct}. Solutions of II_{Ct} below pH 2 are transparent in the visible region. Between pH 3 and 4, the spectra display maxima at 367 nm with shoulders at 400 nm. A plot of the absorbances at 367 nm vs. the pH from 1.5 to 4.0 fits a theoretical sigmoid based on a pK_a value of 2.8. Because exactly the same ionization behavior is seen with the corresponding methyl sulfonate, II_{me}, this pK_a must characterize the ionization of the phenol. Figure 2 reveals that a further increase in the pH results in the loss of the characteristic shoulder at 400 nm. Therefore, it is necessary to analyze both the appearance and the disappearance of the shoulder. A plot of the absorbance at 415 nm against pH yields an asymmetric bell-shaped curve which has a value of zero for the absorbance at low pH. The simplest interpretation of the curve necessitates the postulation of two ionizations which affect the absorbance and of three species, one with the phenol unionized and two with it ionized. The absorbance data at 415 nm over the pH range 4-9 fit a theoretical curve based on eq 2 which describes such a scheme, where A is the observed absorbance and A_1 and A_2 are the absorbances of the species formed in the middle and alkaline pH regions.

A nonlinear least-squares fit of the data to eq 2 yields values of 2.9 and 4.6 ± 0.3 for pK_{A_1} and pK_{A_2}, respectively. Again, the pK_a value of 2.9 must represent the ionization of the dinitrophenol group. However, the second ionization seen has no analogy with the spectral behavior of the model compounds and, therefore, must represent some interaction with the enzyme which alters the environment of at least part of the dinitrophenoxide group at higher pH values.

$$A = \frac{A_1 + A_2K_{A_2}/H}{1 + H/K_{A_1} + K_{A_2}/H} \qquad (2)$$

Kinetics of the Desulfonylation Reaction of 2-Hydroxy-3,5-dinitro-α-toluenesulfonyl-α-chymotrypsin (II_{Ct}). Solutions of III_{Ct} at pH values of 4.5 and 7 show no tendency to desulfonylate or recover activity toward ATME over a period of 48 hr at room temperature. Considering the sensitivity of the assay reaction, we can set an upper limit of 10^{-8} sec^{-1} on the value of the first-order rate constant for reactivation of this sulfonyl enzyme.

In contrast with III_{Ct}, similar solutions of 10^{-6} M II_{Ct} recover up to 70% of their initial activity on standing for 5

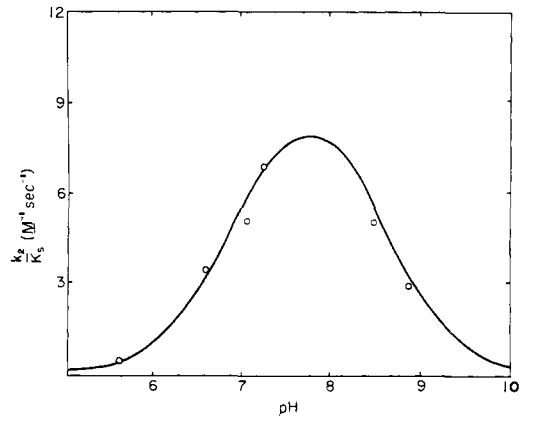


Figure 1. pH profile for the sulfonylation of α-chymotrypsin by β-(2-hydroxy-3,5-dinitrophenyl)ethanesulfonic acid sultone (III) at 25.0°, 0.05 ionic strength, and with 0.45% CH_3CN present. The curve is a theoretical one for pK_1 = 7.01, pK_2 = 8.48, and $(k_2/K_s)_{lim}$ = 11.3 M^{-1} sec^{-1} (a computer-calculated, least-squares fit to the experimental points).

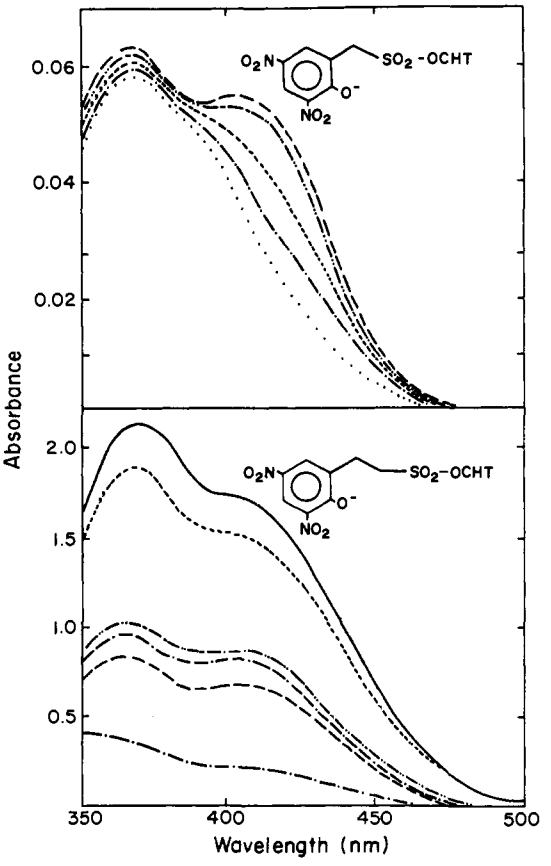


Figure 2. Visible spectra of 4.0×10^{-6} M 2-hydroxy-3,5-dinitro-α-toluenesulfonyl-α-chymotrypsin (II_{Ct}, upper) and 1.4×10^{-4} M β-(2-hydroxy-3,5-dinitrophenyl)ethanesulfonyl-α-chymotrypsin (III_{Ct}, lower) at 25.0°, 0.525 ionic strength, and with 0.05% CH_3CN present. In the upper figure the pH values of the solutions used were (from top to bottom) 3.74, 4.27, 4.74, 4.96, and 8.89. In the lower figure at the top, the spectrum of β-(2-hydroxy-3,5-dinitrophenyl)ethanesulfonic acid in base is given and then in descending order the spectra of solutions of III_{Ct} at pH values of 9.2, 5.13, 4.80, 4.65, and 3.85.

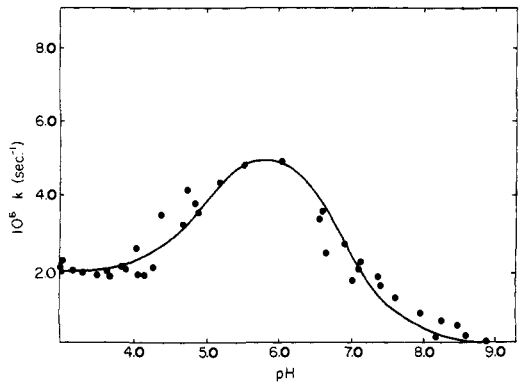


Figure 3. pH profile for the first-order rate constant for recovery of activity by 2-hydroxy-3,5-dinitro-α-toluenesulfonyl-α-chymotrypsin (II_{Ct}) at 25.0°, ionic strength 0.525, and with 0.08% CH_3CN present. The curve is a theoretical one based on eq 4 with $k_{AH_2} = 1.83 \times 10^{-6}$ sec^{-1}, $k_{AH} = 6.05 \times 10^{-6}$ sec^{-1}, $pK_{AH_2} = 5.0 \pm 0.3$, and $pK_{AH} = 6.8 \pm 0.3$ (a computer-calculated, least-squares fit to the experimental points).

days at room temperature. We verified that this recovered activity represents regeneration of unmodified enzyme by determining that both the native (untreated) and reactivated enzyme yield the same value for $K_{m(app)}$ for the reaction with ATME.

We determined the rate constant for reactivation of II_{Ct} from measurements of the initial velocity during the very beginning of the reaction. The low concentration of active enzyme during these measurements minimized problems related to both autolysis of the enzyme and the possible regeneration of II_{Ct} from active enzyme and sultone formed from recyclization.

Solutions of 5×10^{-6} M II_{Ct} were assayed with ATME at various time intervals. Initial rates of reactivation of the enzyme were determined from the slopes of activity vs. time plots. Between pH 3 and 8.5 we observed a linear time dependence for recovery of up to 10% of the initial activity of the unmodified enzyme. Figure 3 shows the pH dependence of the rate constant for reactivation seen with II_{Ct}.

An examination of Figure 3 reveals that the rate constant for reactivation at low pH is pH independent but shows a bell-shaped pH dependency at higher pH. Despite the scatter in the experimental data, which is related to the smallness of these rate constants, we are confident that the rate constant does approach zero in the higher pH range. The desulfonylation reaction minimally appears to involve three species related by two ionizations with only the species present in the lower and middle pH region showing a recovery of enzymatic activity. The simplest scheme which fits these data is shown in eq 4 where AH_2, AH, and A represent various ionic forms of the sulfonyl enzyme II_{Ct}. The pH dependence of the first-order rate constants for the regeneration of active enzyme predicted by the scheme of eq 3 is de-

$$\begin{array}{ccc} AH_2 & \overset{K_{AH_2}}{\rightleftharpoons} & AH \overset{K_{AH}}{\rightleftharpoons} A \\ \downarrow k_{AH_2} & & \downarrow k_{AH} \\ \text{active enzyme} + \text{product} & & \text{active enzyme} + \text{product} \end{array} \quad (3)$$

scribed by eq 4. A nonlinear least-squares computer calcu-

$$k = \frac{k_{AH_2}H/K_{AH_2} + k_{AH}}{1 + H/K_{AH_2} + K_{AH}/H} \quad (4)$$

Table II. Kinetics of Formation and Decomposition of Sulfonyl Enzymes

Sultone	Sulfonylation k_2/K_S, M^{-1} sec^{-1}	Sulfonylation pK_a	Desulfonylation k_{max}	Desulfonylation pK_a
I[a]	3.1×10^5	7.04, 8.64	4.54×10^{-2}	6.96, 7.64
II			6.0×10^{-6}	5.0, 6.8
III	11.3	7.01, 8.48	$<10^{-8}$	

[a] See ref 1 and 3.

lated fit of this equation to the experimental data yields values of $k_{AH_2} = 1.8 \times 10^{-6}$ sec^{-1}, $k_{AH} = 6.0 \times 10^{-6}$ sec^{-1}, $pK_{AH_2} = 5.0 \pm 0.3$, and $pK_{AH} = 6.8 \pm 0.3$.

Discussion

Both of the dinitro-substituted sultones, II and III, react readily with Ct, producing relatively stable sulfonyl enzyme species with covalently attached dinitrophenoxide chromophores. While we did not study the kinetics of the formation of the sulfonyl enzyme II_{Ct}, the continuity of mechanism exhibited in the reaction of a series of substituted 2-hydroxy-α-toluenesulfonic acid sultones with hydroxide ion[7] suggests that the reactions of the five-membered sultones, I and II, with the active site serine hydroxyl in Ct should be similar.

The kinetics of the sulfonylation of Ct by the six-membered dinitrosultone, III, reveal that the pH dependency of the function k_2/K_s is bell-shaped with pK_a values quite similar to those obtained with sultone I (see Table II).[1] Similar results have been obtained with a number of other cyclic esters, including cyclic sulfates, cyclic phosphates, and lactones, which were allowed to react with the active site of Ct.[8] Neither the enlargement of the cyclic ester ring nor the addition of the second nitro group in the aromatic ring affects this pattern of reactivity. There is no evidence in the present work to alter the interpretation of the process of sulfonylation of Ct by sultones as presented in a previous paper from this laboratory.[1] These reactions utilize the catalytic moieties of the enzyme to effect a ring-opening sulfonylation of the active-site serine with concomitant generation of the covalently attached nitrophenol group.

Despite the presumed similarities in the formation of the two sulfonyl enzymes, II_{Ct} and III_{Ct}, their stabilities are quite different. There is no detectable desulfonylation of III_{Ct} in the pH range 4–7. The spectral evidence suggesting that the phenol moiety of III_{Ct} is bound in an hydrophobic region of the enzyme, rather than at the active site, emphasizes the importance of the proximity of the phenolic group and the active site histidine-57 imidazole group for the occurrence of any desulfonylation reaction.

Based on its structural similarity with the other Ct derivatives formed from five-membered sultones such as 2-hydroxy-α-toluenesulfonyl-α-chymotrypsin and I_{Ct} which undergo enzymatic desulfonylation, we felt that it should be possible to see a slow, enzymatic desulfonylation with II_{Ct}. Despite the unusual behavior at low pH, the desulfonylation reaction of II_{Ct} fully supports this premise. The latter enzymatic reaction, however, has a maximum observed rate constant which is nearly 10^4 smaller than the comparable rate constant for I_{Ct}.

The reaction sequence of eq 5 has been shown to fit the results seen in the case of I_{Ct}, where E represents free Ct, S is the sultone, ES is the Michaelis complex, ES′ is the sulfonyl enzyme, P is the product sulfonic acid, and k_{sp} is the pseudo-first-order rate constant measured for the nonenzymatic hydrolysis of S under the given conditions.[3] When the active enzyme, E, generated from I_{Ct} is not scavenged, the

apparent rate of desulfonylation is decreased by resulfonylation (steps K_s and k_2 of eq 5) of E by the sultone produced by recyclization (step k_{-2}). Despite the very low initial concentrations of enzyme and sultone (there is no free sultone at time zero), it is necessary to consider whether the low rate of desulfonylation observed with II_{Ct} can be explained by a similar reaction. On the basis of the scheme of eq 5, assuming $E \ll K_s$, a steady state in S and negligible changes in ES′, it can be shown[9] that significant resulfonylation of E during the desulfonylation process should be accompanied by negative curvature in the [E] vs. time plots. During the course of the desulfonylation reactions of II_{Ct} we observed, the free enzyme concentration changes from less than 2% to about 10% of the total enzyme species present, and this represents a fivefold change in [E]. Therefore, the observation that plots of active enzyme concentration vs. time for the desulfonylation reaction are linear rules out the resulfonylation reaction as the cause for the low rate constant for reactivation of II_{Ct}.

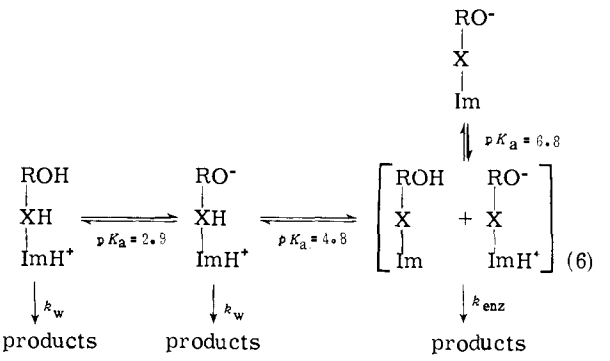
$$E + S \underset{K_s}{\rightleftharpoons} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' \xrightarrow{k_3} P$$

$$S \xrightarrow{k_{sp}} P \qquad (5)$$

The pH independence of the rate of desulfonylation of II_{Ct} observed below pH 4 has no analogy with known deacylation reactions of acyl-Ct species which exhibit a dependence solely on the ionization of a base of $pK_a = 7$ throughout the range of pH 7–2.[10] The rate constant seen in the low pH range for the desulfonylation of II_{Ct} ($k = 1.8 \times 10^{-6}$ sec^{-1}) is only slightly smaller than the rate constant for the aqueous hydrolysis of methyl methanesulfonate ($k_{H_2O} = 5.3 \times 10^{-6}$ sec^{-1}),[11] a reaction in which C–O bond scission occurs. Furthermore, preliminary studies[9] on the aqueous hydrolysis of the model methyl esters I_{me} and III_{me} have indicated that the observed pseudo-first-order rate constants change less than a factor of 2 upon ionization of the phenolic proton. Therefore, both the value of the rate constant for desulfonylation of II_{Ct} and its insensitivity to the state of ionization of the dinitrophenolic group suggest that it may characterize a nonenzymatic displacement by water of the sulfonate group from the β carbon of serine-195.

The correspondence of the pK_a values for the ionization of the dinitrophenol groups in II_{Ct} ($pK_a = 2.9$) and II_{me} ($pK = 2.9$) indicates that at low pH this group in II_{Ct} is exposed to the solvent. Thus, both the dinitrophenol group and the sulfonate ester linkage in II_{Ct} appear to be "out" in the aqueous medium at low pH.

While we can find analogy for the high pH behavior of II_{Ct} in other Ct reactions and a reasonable model for the low pH reaction in the behavior of the methyl esters I_{me}–III_{me}, there appears to be no simple explanation, based on these models, for the transition near pH 4.8 seen in both the spectrophotometric titration and reactivation kinetics of II_{Ct}. We propose the scheme of eq 6 as the simplest explanation of these pH dependencies for II_{Ct} which is warranted by our data. Here X–H represents an enzyme bound functional group with a pK value of approximately 4.8. Its ionization is related to the loss of the shoulder in the absorption spectrum of II_{Ct} at 400 nm. Because the dinitrophenol group is already ionized at the pH values where the ionization of X–H is significant, this must indicate that there is a concomitant charge in the environment of the dinitrophenolate chromophore which is associated with that particular absorbance. In view of the stability of II_{Ct} above pH 8 compared to the rate of its aqueous hydrolysis at low pH, it appears that the ionization of X–H may also reduce the accessibility of the sulfonate ester group to reaction with water. Our results then suggest that the ionization of the enzyme bound group with $pK_a = 4.8$ results in a significant conformational change near the enzyme active site. While the details of this movement are not established, it is clear that when the dinitrophenol ring and at least part of the sulfonylated serine are "out" in the aqueous medium, nonenzymatic desulfonylation predominates. This nonenzymatic reaction, the k_w step of eq 6, is not affected by the state of ionization of the dinitrophenolic group and seems to occur at an equal rate from either of the two species which predominate below pH 4.0. Equation 4 may be obtained from the scheme of eq 6 by making the assumption that the two



(6)

lower pH species desulfonylate at the same rate.

Having considered the origin of the observed pK_a values of 2.9 and 4.8, the assignment of the kinetically determined value of $pK_{AH} = 6.8$ (eq 3) must be discussed next. By analogy with the data in the case of I_{Ct} and in view of the assignments in the cases of the pK_a 2.9 and 4.8 ionizations, we have made the assignment shown in eq 6 for the $pK_a = 6.8$ ionization. Equation 2 can be obtained from the scheme of eq 6 if the assumption is made that those species predominant at higher pH in which X–H is ionized have the same molar absorbance at 415 nm.

Returning now to the reasons for the slower rate of desulfonylation of II_{Ct} as compared to I_{Ct}, the most probable cause for this phenomenon appears to be related either to the reduced basicity of the phenolic nucleophile in II_{Ct} or to a reduction in the formally neutral form of II_{Ct} vs. that of I_{Ct} which is in the reactive state. The respective fractions of Im–ROH and ImH^+–RO^- present in the equilibrium mixture of the formally neutral species can be estimated from the values of K_1 and K_2 (see the scheme of eq 1). The pK assignments just discussed suggest that the ionizations of ROH and ImH^+ are independent of each other for II_{Ct}. Assuming $pK_2 = pK_3$ ($pK_1 = pK_4$) for this sulfonyl enzyme (eq 1), $pK_1 = 2.9$ and $pK_2 = 6.8$. As discussed earlier, the two formally neutral species are present to about an equal extent in I_{Ct} but from the pK_1 and pK_2 values

$$\begin{bmatrix} RO^- \\ | \\ ImH^+ \end{bmatrix} = 7.9 \times 10^3 \times \begin{bmatrix} ROH \\ | \\ Im \end{bmatrix} \text{ in the case of } II_{Ct}$$

From the maximum rate constants observed for the desulfonylation of the corresponding sulfonyl enzymes[3] and the values of pK_1 and pK_2, we calcuate that if [ImH^+–RO^-] were the reactive species $k_{[ImH^+\text{-}RO^-]}$ would be 8.25×10^{-2} sec^{-1} for I_{Ct} and 6.0×10^{-6} sec^{-1} for II_{Ct}. The value of $k_{[Im\text{-}ROH]}$ would be 9.75×10^{-2} for I_{Ct} and 4.8×10^{-2} sec^{-1} for II_{Ct}. From the pK_1 values for I_{Ct} and II_{Ct} the β value for desulfonylation is computed to be 1.1 if ImH^+–RO^- is the reactive species and less than 0.1 if Im–ROH is. There is a paucity of relevant information in the literature

on β values for the reactions of nucleophiles with sulfonyl groups in sulfonate esters. However, the β value measured for the nucleophilic reactions of substituted phenolate species with 2,4-dinitrophenyl *p*-toluenesulfonate is 0.26.[12] If one considers the fact that the β value for the nucleophilic reactions of simple primary and secondary amines with phenyl acetate in aqueous solutions is about 0.72,[13] whereas the β values for the general base catalyzed reactions of sterically related amines with the furoyl ester of serine-195 in α-chymotrypsin are in the range 0.13–0.19,[14] it seems reasonable to expect that the β value in the desulfonylation of the sulfonylchymotrypsins should be lower than 0.26 rather than higher. Therefore, the slow desulfonylation of II_{Ct} is best rationalized on the basis that Im-ROH is the reactive species. Hence, we conclude that the desulfonylation reactions of I_{Ct} and II_{Ct} are mechanistically similar to deacylation reactions of the acylchymotrypsin species in that they require the imidazole ring of histidine-57 to be unprotonated and the deacylating nucleophile to be un-ionized.

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