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Energetics of α-Chymotrypsin-Mediated Hydrolysis of a Strained Cyclic Ester†

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Received April 17, 1986; Revised Manuscript Received September 15, 1986

ABSTRACT: Hydrolysis of *o*-hydroxy-α-toluenesulfonic acid sultone (sultone II) is mediated by α-chymotrypsin. Sultone II is a highly strained cyclic ester substrate that forms a covalent intermediate with the enzyme and is therefore expected to release ring-strain energy upon formation of the sulfonyl enzyme species. It is found that the equilibrium constant for forming the covalent intermediate from the Michaelis complex is quite modest (K_2 = 16.4), suggesting that perhaps the strain energy is not released in the ring-cleavage event. The implied retention of chemical (strain) energy by the sulfonyl enzyme species raises the question of the means by which the enzyme avoids expression of strain energy and the implications of this effect in the catalytic sequence. High-pressure liquid chromatography (HPLC) rate data demonstrate facile reversion of sulfonyl enzyme to the Michaelis complex, and that reversion is preferred over hydrolysis of the covalent intermediate. pH-independent rate and equilibrium constants are derived for the α-chymotrypsin-mediated hydrolysis of sultone II, and pK_a values for groups on the enzyme are reported that are consistent with literature values obtained from analysis of nonspecific substrate hydrolysis by the enzyme.

In numerous cases, the $\Delta G°$ for binding a transition-state analogue to an enzyme has been found to be far more favorable than the $\Delta G°$ for binding the corresponding substrate (Wolfenden, 1972, 1978; Lienhard, 1973). On the basis of these observations as well as others, it has been proposed that the difference in free energy between the $\Delta G°$ values for transition-state analogue–enzyme and enzyme–substrate complex formation is representative of the free energy that can be utilized in bringing about rate acceleration (Jencks, 1975). More specifically, Jencks suggested that the full complement of binding energy is prevented from being realized as observed binding in the ES complex because of requirements for distortion, desolvation, and perhaps other energy-requiring factors (Jencks, 1960, 1975). These destabilizing effects are ultimately removed on reaching the transition state, which is presumed to be the state of most favorable interaction between the enzyme and the species being catalyzed.

The principle behind the proposal is that the potential or expected change in free energy from one part process (e.g., binding) is fully realized only in the subsequent transition state and a portion of the expected free energy does not appear in the observed $\Delta G°$ for binding. Transition-state analogue binding has provided much of the support for this concept with the resulting tendency to focus on noncovalent binding as the source of energy to be utilized. The principle is a general one and is not necessarily restricted to bimolecular (binding) events in enzyme catalysis. All that is necessary for application of

† Supported by U.S. Public Health Service Research Grant GM22300 from the National Institutes of Health.
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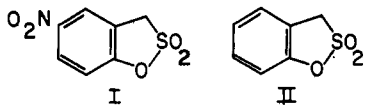
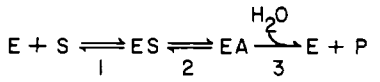
0006-2960/87/0426-0146$01.50/0

Chart I



I II

Scheme I



the principle in any catalytic step is a part process that may serve as a source of free energy. Within an enzyme-catalyzed reaction the two most likely sources of potential free energy for catalytic advantage are binding events and chemical or covalent bond rearrangement steps.

In searching for cases in which the chemical part process may serve as a source of free energy for catalytic advantage, we have looked for examples in which the energy changes of the bond rearrangement events would be emphasized. A potential example described many years ago by Kaiser and coworkers involves the α-chymotrypsin-mediated hydrolysis of sultone I (see Chart I) (Heidema & Kaiser, 1967, 1968, 1970; Izbicka & Bolen, 1981a,b). Crystallographic and alkaline hydrolysis data on sultone I indicate this ester is highly strained, and the kinetics of the α-chymotrypsin-mediated hydrolysis of the compound suggests the reaction can be described by the sequence in Scheme I, with ES representing a Michaelis complex and EA a covalent enzyme–substrate compound [Fleischer et al., 1976; Zaborsky & Kaiser, 1966; Kaiser (1970) and references cited therein].

As a consequence of the high degree of ring strain one might expect that in the transesterification event (step 2, Scheme I) the ring-strain energy released in forming EA would drive the equilibrium of step 2 far to the right. The kinetic data of Kaiser, however, indicate that step 2 is readily reversible (Heidema & Kaiser, 1970). Thus, the $\Delta G°$ for step 2 would appear to be much smaller than expected on the basis of ring-strain release, making this system a worthy candidate for careful thermodynamic study.

Our investigations have focused on sultone II rather than sultone I because the parent sultone is more stable in aqueous solution (Zaborsky & Kaiser, 1970). We have shown that α-chymotrypsin mediates the hydrolysis of sultone II by a mechanism consistent with Scheme I and that the covalent enzyme–substrate compound (EA) can be trapped at low pH (Izbicka & Bolen, 1981a,b). Characterization of sultone II ^{35}S-labeled EA compound was carried out at low pH by SDS–urea denaturation of the protein under reducing conditions, and this was followed by electrophoretic separation of the three peptide chains. The ^{35}S label was found associated with the peptide containing serine-195 and not with the peptide containing histidine-57. This indicates the linkage between sultone II and α-chymotrypsin is a sulfonylserine linkage and that the sultone ring is opened on forming EA.

We have also evaluated the ring-strain enthalpy changes associated with sultones I and II and found them to be very large (Izbicka & Bolen, 1978). Calorimetric heats of alkaline hydrolysis of sultones I and II were found to be 98 kJ/mol more exothermic than those of the corresponding (unstrained six-membered sultones, and we have attributed this enthalpy difference to ring strain. Our previous kinetic studies on the α-chymotrypsin-mediated hydrolysis of sultone II at pH 7 allowed us to estimate an equilibrium constant for step 2, and it was found that EA is favored over ES but only marginally so ($K_2 \sim 10$) (Izbicka & Bolen, 1981b). Thus, in the reaction step in which ring-strain reelease should have caused a large drop in free energy only a very modest energy change occurs. These observations parallel what one might expect for a system in which chemical energy is used in a manner similar to binding energy for providing catalytic advantage.

If the enzyme is operating in this manner, it is essential that the reaction sequence be well-defined. In this paper, we provide a more in-depth study of the reaction sequence, presenting direct evidence for reversion of the EA species to ES and confirming Scheme I as an accurate representation of the enzyme-mediated reaction. An evaluation of rate and equilibrium constants is presented as a function of pH, providing pH-independent rate and equilibrium constants for the reaction sequence.

Materials and Methods

Materials. Three times recrystallized bovine α-chymotrypsin was purchased from Worthington Biochemical Corp. and assayed as 94% active with the use of *N-trans*-cinnamoylimidazole (Schonbaum et al., 1961). Enzyme purity in dilute solution was determined by measuring the rate of hydrolysis of 1.6 mM *N*-acetyl-L-tryptophan ethyl ester (ATrEE) at pH 7.0 in 3.17% acetonitrile and 0.03 M citrate buffer at 25 °C. Active enzyme concentration was calculated by using the Michaelis–Menton equation along with assumed values of k_{cat} and K_m of 26.9 s^{-1} and 9.7 × 10^{-5} M, respectively (Zerner et al., 1964). Concentration of α-chymotrypsin was determined with a molar extinction coefficient of 50 000 cm^{-1} M^{-1} at 280 nm (Bender et al., 1966). Sultone II was synthesized from *o*-hydroxybenzyl alcohol, $POCl_3$, and Na_2SO_3 (Zaborsky & Kaiser, 1966). Proflavin was obtained from commercial sources and recrystallized from water. All other chemicals were of reagent quality.

Spectrophotometric rate measurements were performed on a Beckman Acta M VI spectrophotometer equipped with thermospacers and controlled at 25.0 ± 0.1 °C. High-pressure liquid chromatography (HPLC) analyses were made on a Waters HPLC system equipped with a C_{18} μBondapak column.

Sulfonyl-α-chymotrypsin (EA). EA was prepared by first adding 200 μL of 1 mM α-chymotrypsin at pH 3.0 (no buffer) to 400 μL of 0.15 M sodium citrate buffer at pH 7.0 and readjusting the pH to 7.0 with 1 N NaOH. Sultone II (0.09 M, 50 μL) in CH_3CN was added to the stirred enzyme solution and allowed to stand 15 min at room temperature. The reaction was quenched by adding ca. 20 μL of 1 N HCl to the solution to give a final pH of ca. 3.0. The quenched reaction mixture was placed on a G-25 Sephadex column (1 × 30 cm) equilibrated with pH 3 HCl and eluted with the same solvent to separate sulfonyl enzyme from sultone, CH_3CN, and hydrolysis product, *o*-hydroxy-α-toluenesulfonic acid. The resulting mixture of E and EA was eluted as the first fraction, giving a final protein concentration of ca. 40 μM. The proportion of sulfonyl enzyme in the mixture of E and EA was estimated at ca. 75% as judged by the residual ATrEE activity of the mixture compared to the ATrEE activity present after complete desulfonylation of the sulfonyl enzyme.

For HPLC measurements of desulfonylation, the sulfonyl enzyme was concentrated to about 3 × 10^{-4} M on an Amicon membrane filtration apparatus equipped with a PM 10 membrane filter.

Kinetics. All kinetics except for pH-stat measurements were carried out under the following buffering conditions: 0.03 M sodium citrate for pH 6.5, 0.06 M sodium phosphate for pH 7.0, 7.4, and 7.6, and 0.02 M sodium pyrophosphate for pH 8.0 and 8.4. Ionic strength was adjusted to 0.2 M with NaCl while CH_3CN was adjusted to 4% v/v in all buffering solutions. Temperature was controlled to 25.0 ± 0.1 °C, and

kinetic data were collected and analyzed by using a Bascom-Turner 4120 Data Center.

pH-Stat Measurements. Measurements were performed on a Metrohm pH-stat assembly under a nitrogen atmosphere and maintained at 25.00 ± 0.03 °C with a Sodev thermostat.

Millipore-filtered water used for preparation of all solutions was boiled and degassed. Fresh solutions of NaOH were prepared and standardized daily from carbonate-free saturated stock solution. Sufficient CH_3CN was added to the titrant to give a final concentration of 4% v/v. The enzyme solution containing 0.2 M KCl was adjusted to the pH of interest, and the reaction was initiated by addition of sufficient sultone II stock solution in CH_3CN to give a final CH_3CN concentration of 4% v/v. Several controls were run to assess possible contributions to the pH-stat velocity measurement. The rate of titrant consumption was determined for a blank control consisting of addition of sufficient CH_3CN to a 0.2 M KCl solution to give a final CH_3CN of 4% v/v. Similarly, solvent-mediated hydrolysis of sultone II was evaluated by measuring the velocity of base consumption in a solution containing all components except enzyme. The rate contribution due to inhibited enzyme was determined by carrying out the reaction with (*o*-hydroxyphenyl)ethanesulfonic acid sultone in place of sultone II. (*o*-Hydroxyphenyl)ethanesulfonic acid sultone differs from sultone II in that it binds noncovalently to α-chymotrypsin as well as sultone II but does not sulfonylate the enzyme (Izbicka & Bolen, 1981a). It also does not hydrolyze without the ample presence of strong base (Izbicka & Bolen, 1978). Thus, consumption of titrant in this control may be attributed to autolysis of the inhibited enzyme and any contributions due to solvent blank.

Desulfonylation Kinetics Measurements. Three different procedures were used to monitor desulfonylation kinetics. The *ATrEE slope assay* was performed by adding 50–100 μL of EA solution (unbuffered) at pH 3 to 3.0 mL of a buffered solution containing a saturating concentration of ATrEE and 4% v/v CH_3CN. Actual concentrations in the reaction mixture were 0.1 μM EA and 3 mM ATrEE with a final CH_3CN concentration of 4% v/v. The hydrolysis of ATrEE was then followed with time by observing the increase in absorbance at 300 nm.

The rate of EA decomposition was also determined by measuring the rate of proflavin binding to the free enzyme generated on desulfonylation. The *proflavine displacement assay* involved addition of 100 μL of EA stock solution at pH 3.0 to 0.9 mL of buffered proflavine solution containing 4.44% v/v CH_3CN. This gave reaction mixture concentrations of 6.4 μM EA with 60 μM proflavine and 4% v/v CH_3CN. The reaction was monitored at 465 nm, the maximum wavelength of the enzyme–proflavine complex.

The third method, *HPLC rate assay*, monitored the rate of formation of the EA decomposition products, sultone I and *o*-hydroxy-α-toluenesulfonic acid. HPLC was used to separate and quantitate the reaction product generated by mixing 1.0 mL of buffered aqueous CH_3CN solution with 0.5 mL of concentrated EA stock at pH 3.0 (final CH_3CN of 4% v/v). At given time intervals, 200 μL of the mixture was quenched with 10 μL of 1.3 N HCl and the resultant sample analyzed by HPLC using eluents of 0.06 M sodium phosphate (pH 3.0) with 4% CH_3CN to determine *o*-hydroxy-α-toluenesulfonic acid and 0.06 M sodium phosphate (pH 3.0) with 40% CH_3CN to evaluate sultone II. Detector sensitivities of 0.001 and 0.0005 absorbance at 254 nm were used throughout, and the compounds were quantitated by integration of peak areas using a Bascom-Turner 4120 Data Center.

Sulfonylation Kinetics Measurement. Under conditions with substrate concentration larger than enzyme, burst kinetics were observed by following the increase of absorbance at 280 nm. Pseudo-first-order experimental rate constants (k_{obsd}) were evaluated in the manner described by Bender et al. (1967) and Kezdy and Bender (1962). Analysis of K_2 and k_2 followed the methods of Himoe et al. (1969) and Lobb and Auld (1979) as given under Results.

Eight to ten different sultone concentrations ranging from 3.0×10^{-4} to 1.0×10^{-5} M were used for each pH investigated. Enzyme concentration ranged from 10 to 25 μM.

RESULTS

The central feature of the α-chymotrypsin-mediated decomposition of sultone II is the sulfonyl enzyme species EA. The kinetics can be divided into two experimentally separable phases, the sulfonylation phase, in which EA is formed, and the desulfonylation phase, which monitors the decomposition of EA. Since results for desulfonylation are necessary for analysis of the sulfonylation phase, the data for desulfonylation are presented first.

Desulfonylation. We have previously shown that sulfonyl-α-chymotrypsin (EA) formed at pH 7 can be trapped by rapidly changing the pH to 3 (Izbicka & Bolen, 1981a,b; Nitta et al., 1984). The kinetically trapped intermediate can be chromatographed at pH 3 on G-25 Sephadex to separate it from excess sultone II and the product, sulfonic acid. This intermediate is stable for a matter of several hours at low pH. Approximately 75–85% of the enzyme purified in this manner is sulfonyl enzyme, and the remainder is α-chymotrypsin. Upon rapid titration of this purified EA to the pH of interest, the decomposition of sulfonyl enzyme can be monitored by any of several assay procedures. Two of these assays, proflavine displacement and *N*-acetyl-L tryptophan ethyl ester (ATrEE) slope assay, monitor the appearance of free α-chymotrypsin while the HPLC assay follows the appearance of either sultone II or *o*-hydroxy-α-toluenesulfonic acid product with time.

On addition of an aliquot of the purified EA stock to a solution of ATrEE at saturating (V_m) concentration in the neutral pH range, the velocity will be directly proportional to the amount of competent α-chymotrypsin acting on ATrEE ($V_m^{app} = k_{cat}[E]$). As EA decomposes during the ATrEE assay, more α-chymotrypsin becomes available for catalysis and the ATrEE reaction velocity should show an acceleration ($dV_m^{app}/dt = k_{cat}d[E]/dt$). Figure 1A demonstrates the acceleration predicted, and the first-derivative plot of these data represents the first-order decomposition of EA.

An additional means for evaluating the observed first-order rate constant in this type of assay was developed by Dixon and Neurath and is illustrated in Figure 1B (Dixon & Neurath, 1957). This procedure requires extrapolation of the ATrEE velocity to zero time after all of the EA decomposes to free enzyme. For some pH values, this requirement for a base line created practical problems of maintaining V_m conditions for extended time periods. The first-derivative technique, being amenable to Guggenheim analysis (Guggenheim, 1926), obviates the need for a base line, but this method is, unfortunately, sensitive to the noise level in the absorbance vs. time curve. In practice, the two methods of analysis complement one another, but the Dixon procedure has proved to be the most generally useful of the two.

In terms of Scheme I, EA disproportionates in two directions to form ES as well as free enzyme and *o*-hydroxy-α-toluenesulfonic acid. The ES complex can either revert to EA, via k_2, or dissociate (k_{-1}) to free enzyme and sultone. Once free enzyme is formed, it is immediately sequestered by the pres-

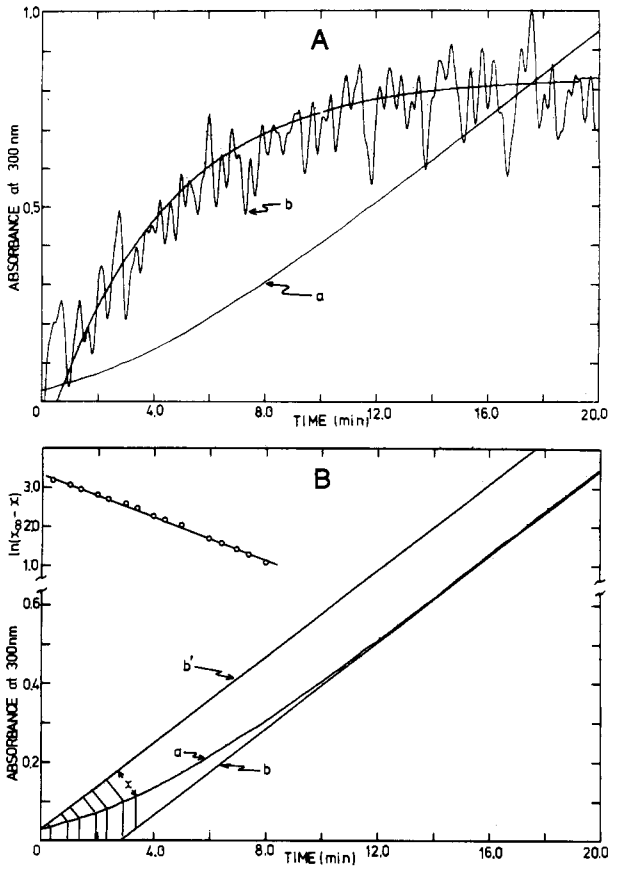
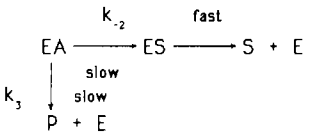


FIGURE 1: (A) Rate of EA desulfonylation using the ATrEE slope assay at 25 °C. (a) Hydrolysis of ATrEE was followed with time by addition of 100 μL of 64 μM purified EA stock at pH 3 to 3.0 mL of 1.4 mM ATrEE in 0.06 M phosphate buffer with ionic strength of 0.2 M adjusted with NaCl. The final pH was 7.03, and the solution contained 4% v/v CH_3CN. (b) First-derivative plot of curve a expanded 15 times. The solid line through the first-derivative data represents a rate constant of 0.004 s^{-1}. (B) Dixon–Neurath treatment of EA desulfonylation using the ATrEE slope assay. (a) Original ATrEE assay data as in panel A(a). (b) Maximum velocity achieved for ATrEE assay after full EA decomposition, extrapolated to t = 0. (b') Line drawn parallel to line b and beginning at the initial absorbance value. The procedure involved constructing chords perpendicular to line b' and intersecting the ATrEE absorbance curve at the times indicated. (c) Upper left corner gives the first-order plot of data determined from use of chords (x and x_∞) with a rate constant of 0.0045 s^{-1}.

Scheme II



ence of saturating ATrEE or proflavine, thus causing enzyme–sultone (ES) dissociation to be irreversible. Dissociation of ES is believed to be much faster than reversion of ES to EA (i.e., $k_{-1} \gg k_2$), and under such conditions the decomposition of EA can be approximated by Scheme II, giving an observed first-order rate constant for decomposition equal to $k_{-2} + k_3$.

The rationale for expecting ES dissociation (k_{-1}) to be of much greater magnitude than sulfonylation (k_2) stems from the fact that small molecule substrates and competitive inhibitors of α-chymotrypsin are known to have second-order association rate constants (k_1) of the order of 10^6–10^8 M^{-1} s^{-1} (Fersht, 1985). Coupling this information with a measured (pH-independent) dissociation constant (K_s) for sultone II of 2.5 mM predicts a k_{-1} of about 2500 s^{-1}. Thus, k_{-1} is a

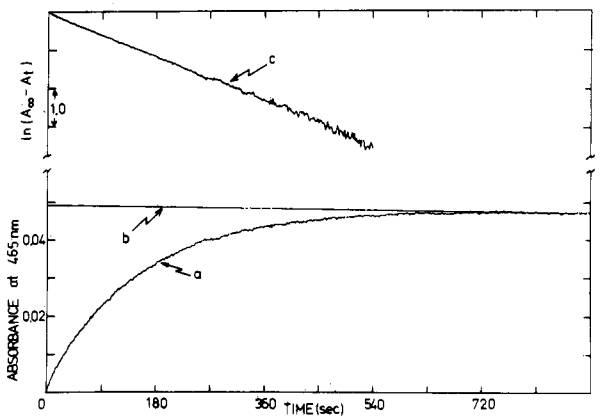


FIGURE 2: Kinetics of EA desulfonylation using the proflavine displacement assay. (a) Proflavine binding was followed with time at 25 °C by addition of 100 μL of 65 μM purified EA stock to 0.9 mL of 66.7 μM proflavine in 0.082 M phosphate buffer with ionic strength adjusted to 0.2 M with NaCl. The final pH was 7.45, and the solution contained 4% v/v CH_3CN. (b) Final absorbance extrapolated to t = 0, representing the A_∞ line. (c) First-order plot of data determined from (a) and (b) with a rate constant of 0.0065 s^{-1}.

minimum of 10 000-fold larger than k_2 or k_{-2}.

The proflavine displacement assay is performed in a manner similar to that of the ATrEE assay except a saturating concentration of proflavine is used in place of ATrEE substrate. The absorbance at 465 nm represents the maximum difference in extinction coefficient between the enzyme–proflavine complex and proflavine alone. The first-order increase in absorbance at this wavelength gives the rate of formation of enzyme–proflavine complex resulting in the same observed (k_{-2} + k_3) rate constant as in the ATrEE assay. A representative example of this assay is given in Figure 2.

HPLC has been used to follow the decomposition of EA by monitoring the appearance of sultone II or *o*-hydroxy-α-toluenesulfonic acid product with time. These rate data were obtained at 25 °C by adding purified EA stock at pH 3 to buffer at a fixed pH in the neutral pH range. Timed aliquots (200 μL) from the incubation mixtures were quenched by rapidly adding the aliquot to a vial containing 10 μL of 1.3 N HCl. The amount of sultone II or sulfonic acid product was determined by HPLC analysis of 20 μL sampled from each quenched aliquot, and an example of this assay is given in Figure 3. HPLC peak areas for sulfonic acid in Figure 3 are close to the limit of quantitation. The estimated error in areas is ±0.1 cm^2. Since saturating proflavine or ATrEE was not used in these experiments, Scheme II may only approximately represent the situation under these conditions. Thus, the relative ratio of sultone II/*o*-hydroxy-α-toluenesulfonic acid represents the ratio of constants k_{-2}/k_3 only to the extent that Scheme II adequately represents the system. Sultone II and *o*-hydroxy-α-toluenesulfonic acid were quantitated by integration of HPLC peak areas and gave a pH-independent ratio of about 20 for sultone II/*o*-hydroxy-α-toluenesulfonic acid. Since the sulfonic acid product measured by way of HPLC is close to the detection limit, k_{-2}/k_3 ratios determined by kinetic measurements described below are considered to be more reliable.

In order to evaluate the k_{-2}/k_3 ratio independently, pH-stat measurements were made to determine k_3 by following the appearance of *o*-hydroxy-α-toluenesulfonic acid under V_m conditions (K_m = 0.11 mM, $K_m \neq K_s$ in the mechanism of Scheme I). Final conditions included 82.4 μM enzyme and 3.54 mM sultone in 0.2 M KCl at 25 °C. Apparent velocities were corrected by control experiments for contributions due to solvent-catalyzed hydrolysis and nonspecific hydrolysis due

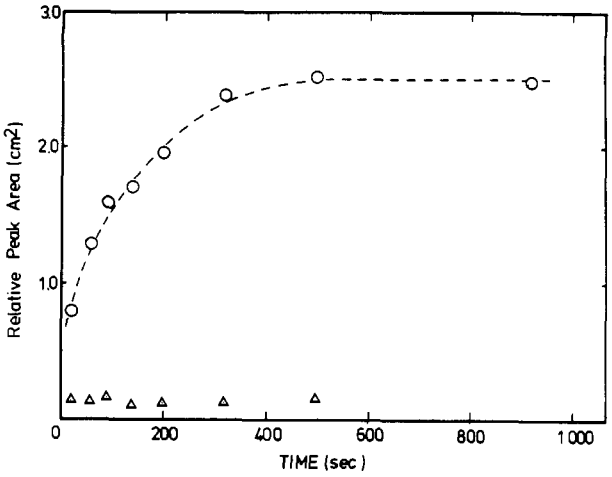


FIGURE 3: Kinetics of EA desulfonylation using the HPLC rate assay at 25 °C. The reaction was initiated by addition of 0.5 mL of concentrated EA (300 μM) to 1.0 mL of 0.11 M phosphate buffer. Ionic strength and acetonitrile concentration were adjusted to 0.2 and 4% v/v, respectively. Aliquots (0.2 mL) of the mixture were quenched with 10 μL of cold 1.3 N HCl at given time intervals, and the resultant samples were analyzed (see text). Amounts of sultone II (○) and *o*-hydroxy-α-toluenesulfonic acid (△) were quantitated by integrating areas under HPLC peaks monitored at 254 nm. The molar absorptivities of sultone II and *o*-hydroxy-α-toluenesulfonic acid are equal at 254 nm. The final pH of the data shown was 7.51, and the dashed line represents a rate constant of 0.0072 s^{-1}.

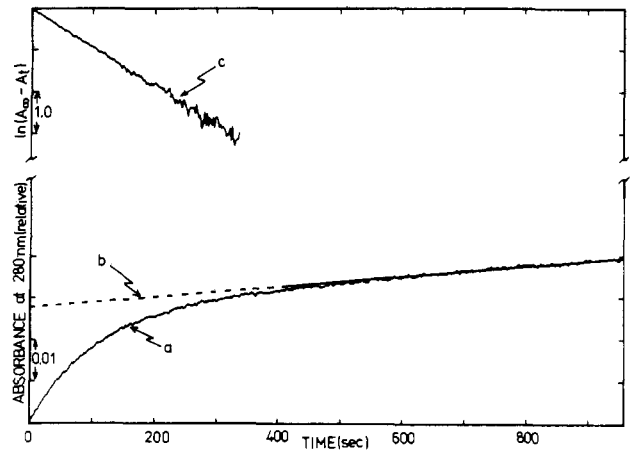


FIGURE 4: Burst kinetics for determination of k_{obsd} at 25 °C. 100 μL of 9.6 mM sultone II in CH_3CN was added to 3.075 mL of 26 μM α-chymotrypsin in 0.062 M phosphate buffer, pH 7.44, 0.8% CH_3CN, and 0.2 M ionic strength. (a) Rate of change at 280 nm. (b) A_∞ line extrapolated to $t = 0$. (c) First-order plot of data derived from (a) and (b) giving a first-order rate constant of 0.0198 s^{-1}.

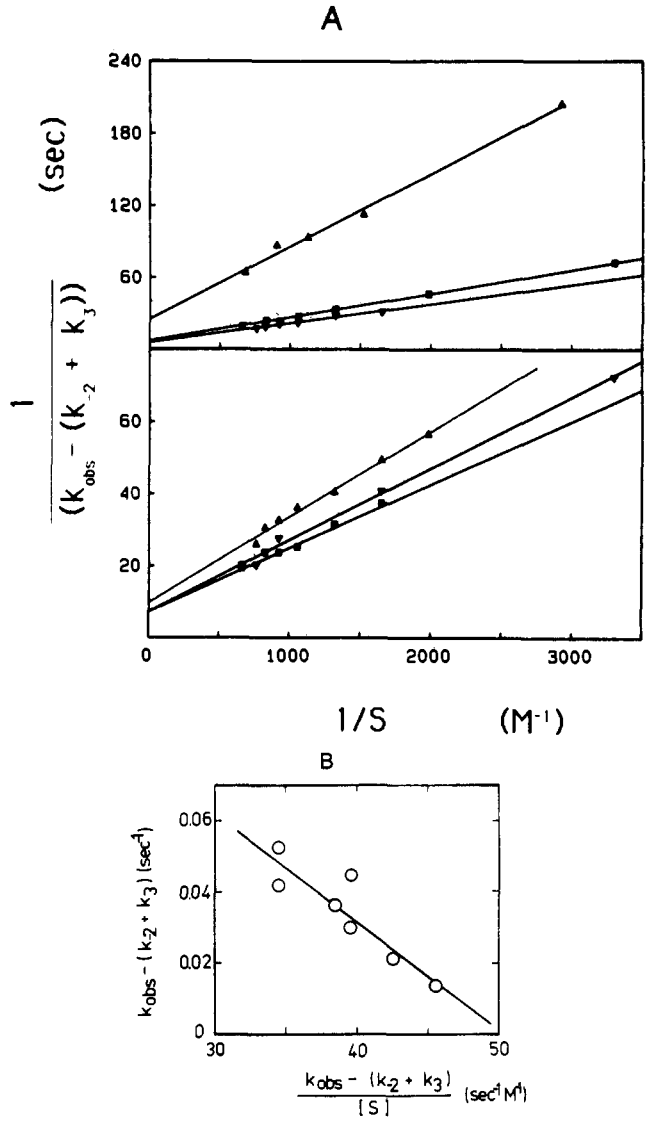


FIGURE 5: (A) Lobb and Auld (1979) plot for estimation of k_2 and K_s for hydrolysis of sultone II by α-chymotrypsin at 25 °C. Reaction mixtures contained 4% CH_3CN in various buffers at a constant ionic strength of 0.2 M. (Upper panel) 0.03 M citrate buffer, pH 6.42 (▲); 0.06 M phosphate buffer, pH 7.44 (■); 0.02 M pyrophosphate, pH 7.94 (▼). (Lower panel) 0.06 M phosphate buffer, pH 7.05 (▲); 0.02 M pyrophosphate buffer, pH 8.40 (▼); 0.06 M phosphate buffer, pH 7.65 (■). Lines represent linear least-squares fits to the data; k_{obsd} values were derived from proflavine displacement data (see Materials and Methods). (B) Representative plot for evaluation of k_2 and K_s at pH 7.44 by the procedure of Himoe et al. (1969).

to inhibited enzyme. The corrected reaction velocity divided by enzyme concentration gave k_{cat} as a function of pH, and this was related to k_3 by the relationship $k_{cat} = k_2k_3/[(k_{-2} + k_3) + k_2]$ derived from steady-state kinetic analysis of Scheme I. Knowledge of the pH dependence of k_{cat} and $k_{-2} + k_3$, along with k_2 obtained from experiments to be described, gave k_3 as a function of pH. The rate constant k_{-2} was evaluated by subtraction of k_3 from the observed first-order desulfonylation rate constant ($k_{-2} + k_3$), and the ratio k_{-2}/k_3 was obtained as a function of pH and is presented later as part of Figure 6.

Sulfonylation. Figure 4 demonstrates the burst kinetics observed at 280 nm on mixing sultone II with α-chymotrypsin. This wavelength gives the maximum absorbance difference between sulfonyl enzyme and the other components in solution. The observed first-order rate constant was determined from burst kinetic data in the usual manner (Heidema & Kaiser, 1970; Himoe et al., 1969) and was related to the mechanism of Scheme I by simple extension of a derivation by Bender et al. (1967) as given in eq 1. Lobb and Auld have also used

$$k_{obsd} = (k_{-2} + k_3) + k_2[S]/(K_s + [S]) \qquad (1)$$

eq 1 for analysis of a carboxypeptidase-catalyzed reaction (Lobb & Auld, 1979). With knowledge of k_{obsd} from burst kinetics, and $k_{-2} + k_3$ for desulfonylation data, eq 1 can be rearranged and plotted for evaluation of k_2 and K_s. Parts A and B of Figure 5 were obtained by using the different plots.

pH Dependence. Figure 6 gives the pH dependence of k_{-2}/k_3 as determined from k_3 data obtained from pH-stat measurements and average k_{-2} values evaluated from ATrEE slope and/or proflavine displacement assays. The ratios are virtually constant with pH, with k_{-2} being about 10-fold larger than k_3. A ratio of this magnitude demonstrates that the observed first-order rate constant for EA decomposition (k_{-2}

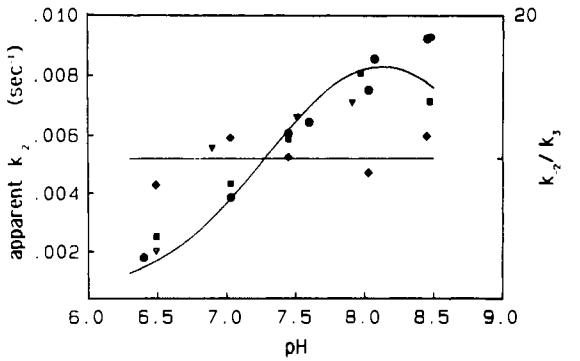


FIGURE 6: Dependence of k_{-2}/k_3 and (also) apparent k_{-2} vs. pH at 25 °C. Ratios of k_{-2}/k_3 were obtained from use of pH-stat measurements of k_3 along with k_{-2} from desulfonylation experiments (♦). Apparent k_{-2} values were determined from the ATrEE slope assay (■), proflavine displacement assay (●), and HPLC rate assay (▼). The solid line represents the best fit of these data to eq 2 to give limiting values of 7.35 ± 0.13 and 0.011 ± 0.001 for pK''_{a1} and k_{-2}, respectively. HPLC rate assay data were not used for the data fitting.

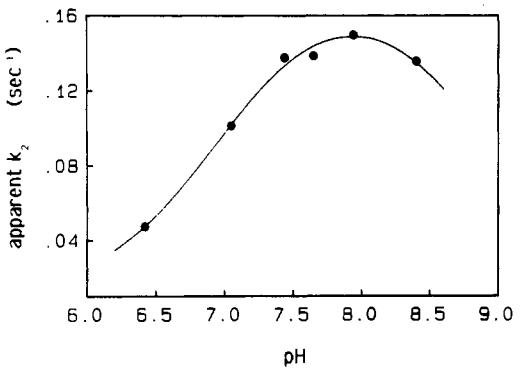


FIGURE 7: pH dependence of k_2 apparent data derived from burst kinetic studies at 25 °C. These data were fitted to eq 3, giving limiting values of 6.96 ± 0.04 and 0.180 ± 0.004 s^{-1} for pK'_{a1} and k_2, respectively.

+ k_3) essentially reflects k_{-2}. The pH dependence of the apparent k_{-2} rate constants is also given in Figure 6 and appears to implicate involvement of a group on the enzyme with a pK_a in the neutral pH range.

The apparent k_2 data derived from sulfonylation kinetic data are also markedly dependent on pH as seen in Figure 7. By contrast, K_s values and K_2 equilibrium constants involve a more complex relationship with pK_a's of enzyme functional groups. The pH dependence of K_s is given in Figure 8.

Discussion

A study of the α-chymotrypsin-mediated hydrolysis of sultone II was undertaken to establish the reaction sequence and define the relevant pH-independent rate and equilibrium constants. All of the kinetic data presented are consistent with the sequence given in Scheme I, and HPLC analysis of EA decomposition gives direct evidence for reversion of sulfonyl enzyme (EA) to sultone II plus *o*-hydroxy-α-toluenesulfonic acid. Sulfonyl enzyme accumulates in the steady state, and equilibrium of step 2 is established as a consequence of k_{-2} exceeding k_3 by about 10-fold. These characteristics hold over the pH range investigated.

Since the extent of reversibility of step 2 is of conceptual importance in this system, it is appropriate to define the equilibrium in terms of pH-independent rate and equilibrium constants. Furthermore, the unusual pH dependence found for α-chymotrypsin-mediated sultone I hydrolysis (Heidema & Kaiser, 1968, 1970), a close structural analogue of sultone II, established a need for a thorough investigation of the kinetic

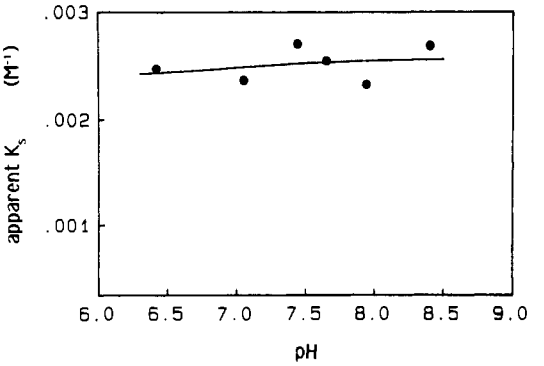
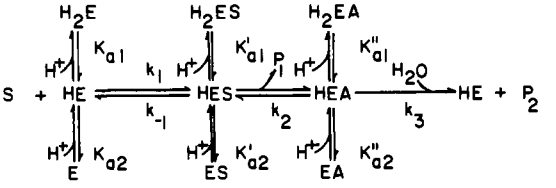


FIGURE 8: pH dependence of K_s apparent data. These data were fitted to eq 4 by taking $pK_{a2} = pK'_{a2} = 8.9$, and $pK'_{a1} = 6.96$. The best fitted line given in this figure resulted in limiting values of 2.51 ± 0.15 mM and 6.93 ± 0.07 for K_s and pK_{a1}, respectively.

Scheme III



and equilibrium properties of the enzyme-mediated hydrolysis of sultone II.

Figures 6–8 demonstrate that, in contrast to sultone I, sultone II hydrolysis exhibits pH profiles reminiscent of a number of specific and nonspecific substrates. A minimal mechanism to describe the pH dependence is presented in Scheme III.

The basis of this scheme stems from pH-dependent profiles of k_2 and k_2/K_s for a variety of substrates which show two ionizable groups on the enzyme with apparent pK_a's in the range of 6.8 ± 0.3 and 8.9 ± 0.3, as well as an apparent pK_a in the range of 7.0 ± 0.3 for k_3 (Bender et al., 1965, 1966). Interpretations of these pH effects have generally been in terms of the 6.8 ± 0.3 pK_a representing the histidine-57 imidazole in the free enzyme, which becomes perturbed to higher values (viz. 7.0 ± 0.3) in the covalent enzyme–substrate intermediate (Bender et al., 1965, 1966; Bender & Kezdy, 1964). In the case of nonspecific substrates (of which sultone II is classified), the imidazole group can be perturbed to values as high as 7.7 (Keizer & Bernhard, 1966). The pK_a of 8.9 ± 0.3 is generally agreed to represent the isoleucine-16 α-amino group involved in electrostatic interaction with aspartate-194 (Keizer & Bernhard, 1966; Fersht & Requena, 1971).

Our analyses of the pH dependence of sultone II hydrolysis are basically described by Scheme III except there is no release of P_1 in step 2, and allowance for reversion of step 2 (k_{-2}) is included. We have assumed K_{a2}, K'_{a2}, and K''_{a2} are equivalent, with a value of 1.26×10^{-9} M ($pK_a = 8.9$). This assumption was made since we were unable to experimentally investigate the full pH range and other enzyme–substrate intermediates do not seem to perturb this pK_a from what it is in the free enzyme (Keizer & Bernhard, 1966).

Decomposition of sulfonyl enzyme is given by $k_{-2} + k_3$, with k_{-2} being the dominant term by about 10-fold. Therefore, the expression for k_{-2} (apparent) as a function of hydrogen ion concentration is given by eq 2. Using K''_{a2} derived from pK''_{a2}

$$k_{-2}^{app} = k_{-2}/(1 + K''_{a2}/[H^+] + [H^+]/K''_{a1}) \quad (2)$$

= 8.9 and fitting the data of Figure 6, we obtained limiting values of 7.35 ± 0.13 and 0.011 ± 0.001 s^{-1} for pK''_{a1} and k_{-2}, respectively. Equation 3 gives the pH dependence of k_2 ap-

$$k_2^{app} = k_2/(1 + K'_{a2}/[H^+] + [H^+]/K'_{a1}) \quad (3)$$

parent with $pK'_{a2} = 8.9$. The best fitted values of pK'_{a1} and k_2 were determined from the data in Figure 7 to be 6.96 ± 0.04 and 0.180 ± 0.004 s^{-1}. Similarly, values of 6.96 and 8.9 for pK'_{a1} and $pK'_{a2} = pK_{a2}$ were assumed in evaluating the limiting values of 2.51×10^{-3} ($\pm 0.15 \times 10^{-3}$) and 6.93 ± 0.07 for K_s and pK_{a1}, respectively, by fitting the data to eq 4 (see

$$K_s^{app} = K_s(1 + K_{a2}/[H^+] + [H^+]/K_{a1})/(1 + K'_{a2}/[H^+] + [H^+]/K'_{a1}) \quad (4)$$

Figure 8). The apparent K_s values obtained from data in Figure 5A were observed to be essentially independent of pH, requiring that the hydrogen ion dependent numerator of eq 4 be approximately equal to the denominator. These results show that the pK_a of around 7 in the free enzyme remains unperturbed on sultone binding to the enzyme. The pH-independent rate and equilibrium constants can be summarized as follows: $k_2 = 0.180 \pm 0.004$ s^{-1}, $k_{-2} = 0.011 \pm 0.001$ s^{-1}, $K_2 = 16.4$, and $K_s = 2.51 \times 10^{-3}$ ($\pm 0.15 \times 10^{-3}$) M.

The pH profile for sultone II hydrolysis by α-chymotrypsin contrasts markedly with that of the nitro derivative, sultone I (Heidema & Kaiser, 1968, 1970). The rationale for the differences is that the nitrosultone generates a EA species with a pK_a in the range of 6.7 whereas the EA species from sultone II has a pK_a of around 10, well outside the pH range investigated. Consequently, sultone II gives a pH dependence much like that of other nonspecific substrates.

Step 2 is the point of focus in this reaction because it involves covalent bond changes between the substrate and enzyme. Formation of sulfonyl enzyme (EA) species results in opening the sultone ring, and as a result of the large degree of ring-strain enthalpy associated with this substrate, a large drop in free energy is expected in going from ES to EA (Izbicka & Bolen, 1978). From the modest value of $K_2 = 16.4$, it appears that the free energy change for ES to EA is much smaller than expected. It is of interest to know whether the modest equilibrium constant is unusually small for this type of transesterification and whether the low value is of mechanistic importance in the hydrolysis of sultone.

One means of assessing the characteristics of sultone transesterification to serine-195 of the enzyme would be to compare this event with a (model) reaction between sultone and a primary alcohol as represented in eq 5. By comparison of the

$$\text{sultone I or II} + \text{ROH} \leftrightarrow \text{sulfonate ester} \quad (5)$$

equilibrium constant of the model reaction with that of ES ↔ EA, it should be possible to determine whether sulfonylation is unusual in the reaction involving enzyme, relative to that of the unconstrained model reaction.

Using primarily sultone I, Deacon et al. (1978) have presented an extensive study of reactions of the type illustrated in eq 5 and have obtained a Brønsted relationship governing the forward and reverse rate constants for transesterification. From log k_2 and log k_{-2} vs. pK_a plots, an equilibrium constant of about 3×10^4 M^{-1} may be calculated for the reaction between the (protonated) primary alcohol, trifluoroethanol, and sultone I.

This model reaction can be compared with the ES ↔ EA event for the α-chymotrypsin-mediated hydrolysis of sultone I, which is reported to have an equilibrium constant (K_2) for sulfonylation of 4×10^3 (Kezdy & Kaiser, 1970). The enzyme-mediated reaction is a factor of 8 less favorable than the model reaction between sultone I and trifluoroethanol. A cratic entropy correction, accounting for the arbitrary choice of the molarity scale in constructing equilibrium constants for bimolecular and unimolecular reactions, results in the model

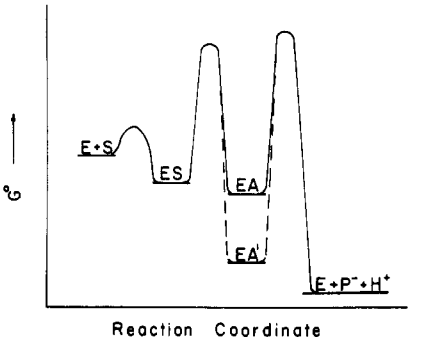


FIGURE 9: Standard-state free energy diagram for reaction between sultone II and α-chymotrypsin. Relative free energy values of E + S, ES, and EA as well as activation free energy barriers for ES → EA, EA → ES, and EA → E + P^- + H^+ were derived from equilibrium and rate constant data determined in this work. EA′ represents a hypothetical sulfonyl enzyme species in which the ring-strain energy has been fully released.

reaction being more favorable by an additional factor of 55. Thus, in unitary free energy terms, the equilibrium constant of the bimolecular model reaction is more favorable than the unimolecular enzyme-mediated event by a factor of more than 400. This result is opposite to that observed in unstrained model systems, where it is found that intramolecular reactions typically have equilibrium constants 10^5-fold larger than the corresponding bimolecular reaction (Jencks, 1975). Since both sultones (I and II) exhibit a large exothermic ring-strain enthalpy change on ring opening, it is presumed that the ES ↔ EA event is less favorable than the model reaction because strain is not completely released. The comparatively modest K_2 does not appear to compromise catalysis since sultone I is a rather good substrate for α-chymotrypsin with a k_{cat}/K_m of 10^5 s^{-1} M^{-1}.

The inference of free energy being less than expected has been proposed for the binding of substrates to enzymes and used to suggest that a portion of the potential free energy of binding is utilized to destabilize the noncovalently bound substrate relative to the subsequent transition state in the reaction sequence (Jencks, 1975). If this principle is also applicable to the intramolecular reaction ES ↔ EA, a catalytic advantage should be realized in step 3 of Scheme I. Figure 9 illustrates how the relative free energy for EA might affect the reaction standard state free energy diagram. The dashed line represents the case expected for a large release of energy on forming EA′ while the solid line gives the free energy relationship between E + S, ES, and EA established in this work. From a kinetic standpoint the advantage of not expressing the full complement of energy is evident from the smaller activation energy needed for EA hydrolysis in comparison with the case in which the energy is expressed, i.e., EA′. The free energy diagram of Figure 9 affords a plausible explanation of the large difference in rates of hydrolysis of EA and (phenylmethanesulfonyl)chymotrypsin (PMS-Ct). Sultone II derived EA differs chemically from PMS-Ct only by having an *o*-hydroxyl group on the phenyl ring. However, EA undergoes hydrolysis with a rate constant of 0.02 min^{-1} at pH 7 while PMS-Ct is stable for weeks under these conditions. If we assume these enzyme structural analogues go through the same transition state for hydrolysis, the differences in their hydrolysis rate would be reflected in differences in their ground-state free energies. Thus, PMS-Ct may be less constrained by the enzyme and have a free energy closer to that of EA′ as opposed to EA as seen in Figure 9.

Since the entropic advantage is substantial for intramolecular reactions compared to bimolecular events, the greater

the ability of the enzyme to constrain the translational and rotational degrees of freedom of reactants in the proper positions to form products or transition state, the closer the enzyme will come to achieving maximum rate enhancement. In the case of ES to EA conversion involving sultone, any rate enhancement in the forward direction due to entropic constraint must also apply to a certain extent to the reversion of EA to ES since the reverse reaction is also an intramolecular event. One can envision the EA species constrained such that the *o*-hydroxyl group is held within proximity of the sulfonylserine linkage to promote reversion of EA and reduce the magnitude of the ES to EA equilibrium constant. Constraints of this type have been proposed by Milstein and Cohen (1970) to explain rate accelerations of 10^{11} in lactonization of rigidly constrained methylated hydrocoumarinic acid as compared with the unconstrained system. Danforth et al. (1976) demonstrated that the effect of freezing conformations, without simultaneously introducing van der Waals repulsions, accounts for a factor of 10^4 acceleration toward lactonization in this system while theoretical calculations by Winans and Wilcox (1976) suggested that steric compression of the *o*-hydroxyl with the carboxyl group accounted for the remaining factor of 10^7. If the enzyme is able to constrain the sultone transesterification such that steric compression of the *o*-hydroxyl and sulfonyl moiety occurs, or the functional groups are simply fixed in close proximity without compression, the magnitude of K_2 would be smaller than expected.

There are several ways in which sulfonylation of enzyme may give a smaller than expected K_2. Among the more obvious possibilities are as follows: (1) the serine-195 hydroxyl of the enzyme may have less potential to react with the sultone than does the alcohol in the unconstrained model reaction, and this could result in a reduction of the expected equilibrium constant; (2) the distribution of productive to nonproductive binding of sultone may significantly dictate the observed ratio of [EA]/[ES] regardless of any driving force due to ring-strain release; (3) there may be chemical coupling in the transesterification event, with some energy being conserved within the sulfonyl enzyme species. There are no easy ways to distinguish among the possibilities, so it is not clear whether the ring-opening event in forming EA acts by inducing a measure of strain or destabilization into the enzyme, resulting in catalytic advantage in subsequent steps of the reaction sequence. It is clear, however, that sulfonylation of the enzyme (k_2) by either sultone I or sultone II is significantly faster than the rate of sulfonylation in the corresponding model reaction with protonated alcohol. It also appears, in the case of sultone I reaction with α-chymotrypsin, that the equilibrium constant for sulfonylation is unexpectedly smaller than the equilibrium constant of the model reaction involving the primary alcohol (trifluoroethanol) and sultone I. The origin of these effects involving sultones I and II is under investigation.

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