Reaction of 28 with Methyllithium. Cyclization of **28** with methyllithium was performed according to the procedure previously described for the protio analog.[1] There was obtained in 60% combined yield a mixture of **29** (66%) and **30** (34%) which were readily separable by preparative vpc (column G,[30] 100°).

For **29**: $\delta_{TMS}^{C_6D_6}$ 3.40–3.00 (m, 1, H_6), 3.17 (s, 3, OCH_3), 2.08 (br s with fine splitting, 1, H_5), 1.85–1.20 (m, 4, methylenes), 1.49 (s, 3, CH_3), and 1.13 (br s with fine splitting, 1, H_7). For $C_9H_{13}OD$ *m/e* 139.1105 (calcd *m/e* 139.1107).

For **30**: $\delta_{TMS}^{C_6D_6}$ 3.62 (d, J = 7 Hz, 1, H_2), 3.02 (s, 3, OCH_3), 2.50 (v br d, J = 7 Hz, 1, H_5), 1.02 (s, 3, CH_3), and 2.3–1.4 (m, 5). For $C_9H_{13}OD$ *m/e* 139.1105 (calcd *m/e* 139.1107).

***syn*-3-Methoxy-1-methyltricyclo[4.1.0.0^{2,7}]heptane-7-*d* (39).** An anhydrous solution of sodium methoxide in methanol-*O*-*d* was prepared by dissolving sodium (300 mg, 13 mg-atoms) in methanol-*O*-*d* (7 ml), and an aliquot (3 ml) of this stock solution together with **21** (295 mg, 2.14 mmol) was sealed in a base-washed glass tube under vacuum. After heating at *ca.* 135–140° for 66 hr, the cooled reaction mixture was treated with water (15 ml) and extracted three times with 10-ml portions of pentane. The combined pentane layers were dried and concentrated by slow distillation using a 6-in. Vigreux column to give a residual oil which was subjected to preparative vpc (column G,[30] 100°). There was isolated a 41% yield of **39**: $\delta_{TMS}^{C_6D_6}$ 3.35–3.02 (m, 1, H_3), 3.22 (s, 3, OCH_3), 2.39 (apparent dd, 1, H_2), 2.08 (m, 1, H_6), 1.90–0.98 (m, 4, methylenes), and 1.50 (s, 3, CH_3). Absence of detectable absorption at *ca.* δ 1.40 confirmed that virtually complete deuteration of the 7-bridge position had occurred. From mass spectral data, deuterium incorporation into **39** was calculated to be $\geq$96% complete. For $C_9H_{13}OD$ *m/e* 139.1105 (calcd *m/e* 139.1107).

For **42/43**, the pmr spectrum (in C_6D_6) was very similar to that previously reported for the unlabeled mixture[1] except that no detectable olefinic absorption was seen in the δ 5.5 region. For $C_9H_{13}DO$ *m/e* 139.1109 (calcd *m/e* 139.1107).

These pmr features contrast with those recorded for the **31/32** mixture obtained from the rearrangement of **29**: $\delta_{TMS}^{C_6D_6}$ 5.47 (br s with additional fine splitting, 1, olefinic), 3.50–3.30 (2 m in 20:80 ratio, 1, H_2), 3.25–2.85 (m, 1, bridgehead), 3.10 (s, 3, OCH_3), 2.15–1.15 (m, 4, methylenes), and 1.44 (t, J = 1.5 Hz, 3, CH_3). For $C_9H_{13}DO$ *m/e* 139.1105 (calcd *m/e* 139.1107).

Acknowledgment. The partial financial support of the National Science Foundation is gratefully acknowledged.

Kinetics of Inactivation of α-Chymotrypsin with Substituted Benzenesulfonyl Fluorides

J. T. Gerig* and D. C. Roe

Contribution from the Department of Chemistry, University of California, Santa Barbara, Santa Barbara, California 93106. *Received August 2, 1973*

Abstract: The inactivation of α-chymotrypsin by a series of fluoro- and trifluoromethyl-substituted benzenesulfonyl fluorides is described. A technique based upon displacement of the dye Biebrich Scarlet from its complex with the enzyme was used to follow the kinetics of the inactivation reaction; in favorable systems this method gives both the binding constant of the irreversible inhibitor (K_I) and the rate constant for covalent bond formation (k_r) in a single experiment. These data are presented and discussed.

Recent nmr studies of the interaction of α-chymotrypsin (CT) with competitive inhibitors have yielded new information regarding the nature of the complexes formed.[1-9] These various applications of nmr techniques to the study of the interactions between CT and substrate-sized molecules suggest that nmr methods might profitably be employed in examination of the acylenzyme intermediate thought to be involved in CT catalysis.[10,11] Many acylchymotrypsins are not stable enough at ordinary temperatures to be observed conveniently in nmr experiments although a recent report suggests that the prospects for these experiments could be improved by operating at subzero temperatures in mixed solvents.[12] However, sulfonyl fluorides have been shown to yield acylenzyme analogs that are stable for long periods of time except at high pH,[13-16] and one of these derivatives, tosylchymotrypsin, has been studied by X-ray crystallographic methods.[17,18] With the hope that nmr studies of the corresponding proteins in solution may ultimately be compared to crystal structure data we have prepared a series of fluoro- and trifluoromethyl-substituted benzenesulfonyl fluorides designed to probe the hydrophobic binding site of CT.[15] The synthesis of these materials and a study of the kinetics of their reactions with this enzyme are reported here; a subsequent paper will deal with nmr studies of the derivatized enzymes.[19]

(1) T. McL. Spotswood, J. M. Evans, and J. H. Richards, *J. Amer. Chem. Soc.*, **89**, 5052 (1967).
(2) E. Zeffren and R. E. Reavill, *Biochem. Biophys. Res. Commun.*, **32**, 73 (1968).
(3) B. D. Sykes, *Biochem. Biophys. Res. Commun.*, **33**, 727 (1968).
(4) J. T. Gerig, *J. Amer. Chem. Soc.*, **90**, 2681 (1968).
(5) B. D. Sykes, *J. Amer. Chem. Soc.*, **91**, 949 (1969).
(6) J. T. Gerig and J. D. Reinheimer, *J. Amer. Chem. Soc.*, **92**, 3146 (1970).
(7) (a) K. L. Gammon, S. H. Smallcombe, and J. H. Richards, *J. Amer. Chem. Soc.*, **94**, 4573 (1972); (b) S. H. Smallcombe, K. L. Gammon, and J. H. Richards, *ibid.*, **94**, 4581 (1972); (c) S. H. Smallcombe, B. Ault, and J. H. Richards, *ibid.*, **94**, 4585 (1972).
(8) J. T. Gerig and R. A. Rimerman, *J. Amer. Chem. Soc.*, **94**, 7549, 7558, 7565 (1972).
(9) B. C. Nicholson and T. M. Spotswood, *Aust. J. Chem.*, **26**, 135 (1973).
(10) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. I, W. A. Benjamin, New York, N. Y., 1966, Chapter 2.
(11) G. P. Hess, *Enzymes, 3rd Ed.*, **3**, 213 (1971).
(12) A. L. Fink, *Biochemistry*, **12**, 1736 (1973).
(13) D. E. Fahrney and A. M. Gold, *J. Amer. Chem. Soc.*, **85**, 997 (1963).
(14) A. M. Gold, *Methods Enzymol.*, **11**, 706 (1967).
(15) D. M. Blow, *Enzymes, 3rd Ed.*, **3**, 185 (1971).
(16) H. Weiner, W. N. White, D. G. Hoare, and D. E. Koshland, Jr., *J. Amer. Chem. Soc.*, **88**, 3851 (1966).
(17) J. J. Birktoft, B. W. Matthews, and D. M. Blow, *Biochem. Biophys. Res. Commun.*, **36**, 131 (1969).
(18) J. J. Birktoft and D. M. Blow, *J. Mol. Biol.*, **68**, 187 (1972).
(19) After this work had been initiated, Dr. A. S. V. Burgen informed us of some preliminary fluorine nmr studies of 4-trifluoromethylbenzenesulfonylchymotrypsin in his laboratory (Sept 1971).

Results

The reaction of benzenesulfonyl fluorides with CT probably follows the scheme

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k_r} \widehat{EI} \quad (1)$$

where E is the enzyme and I is the irreversible inhibitor.[13,14,20] The symbol $\widehat{EI}$ represents the catalytically inactive enzyme formed by internal reaction of the noncovalent complex, EI. A method based on the observation[21] that the dye Biebrich Scarlet (BS) gives a characteristic difference spectrum in the presence of CT, and is displaced from its complex with the enzyme by substrates, competitive inhibitors, and active site reagents, was used to follow the kinetics of the reaction between the sulfonyl fluorides used in this work and α-chymotrypsin. The displacement may be followed in the visible region of the spectrum at λ_{max} of the complex and, in the case of reaction with irreversible inhibitors, is observed to take place in two stages: (1) a rapid initial decrease which occurs immediately after addition of the inhibitor and is related to the K_I of the inhibitor, followed by (2) a slower decay to zero absorbance related to k_r.

Purification of Biebrich Scarlet. Several inconsistencies in the literature forced us to undertake extensive purification of the dye before use. Crude Biebrich Scarlet (C.I. Acid Red 66 from Matheson Coleman and Bell) was observed to have a molar extinction coefficient (ϵ, M^{-1} cm^{-1}) of 26,700 in water at 505 nm. Glazer[21] and Winkelman and Spicer[22] report ϵ_{505} 30,000 and 29,000, respectively; however, Rossi, *et al.*,[23] observed a low value of ϵ due to a 25% impurity (based on Glazer's extinction coefficient) which was not removed by recrystallization from *N*,*N*-dimethylformamide–ether. Jayaram and Rattee[24] recrystallized chromatographically pure Biebrich Scarlet from water by salting out with sodium acetate. They observed ϵ 33,595 at 505 nm, and noted that the dye was 98% pure as judged by elemental analysis. We decided to purify our sample of the dye by the method of these latter authors, with the modification that salting out was accomplished with potassium acetate which is roughly twice as soluble as the sodium salt. Five times recrystallized Biebrich Scarlet gave ϵ_{505} 35,900 and this sample of the dye was used in all of the experiments involving chymotrypsin described below. Eight times recrystallized dye gave ϵ_{505} 35,700 and its was concluded that the material had been purified to constant extinction coefficient.

Exploratory experiments were performed to define the medium dependence of the extinction of the dye. The effects of pH, ionic strength, and organic cosolvent on ϵ_{505} are summarized by the following. Biebrich Scarlet has been shown to have the same value of ϵ_{505} at pH 5.2 and pH 9.5.[22] Preliminary experiments with the crude dye indicated that ϵ was 10% lower in 0.1 *M* phosphate buffer (pH 7.6) than in water alone (pH 10.1). With purified dye, ϵ decreased *ca.* 2% in 0.05 *M* phosphate buffer (pH 7.0) when compared with the value in water, and increased *ca.* 6% over the value in buffer alone when the buffered solution contained 15% (v/v) 2-propanol. It was also noted that solutions of the dye appeared to be quite stable; solutions which had been exposed to the light of the laboratory for over 1 year showed no decrease in ϵ_{505}.

Binding of Biebrich Scarlet to Chymotrypsin. When Biebrich Scarlet in 0.05 *M* phosphate buffer (pH 7.0) was titrated with chymotrypsin, the difference spectra obtained were characterized by a maximum ΔOD at 550 nm and an isosbestic point at 515 nm. After correction for the percentage of active sites in our enzyme preparation,[25] the titration data conformed to a theoretical curve calculated for a 1:1 complex with a dissociation constant, $K_D = 2.26 \times 10^{-5}$ *M* and $\Delta\epsilon_{550}$ = 12,365 (Table I). Glazer has indicated that the dye may bind weakly to other sites on the protein;[21] these secondary interactions were neglected in these calculations and in the subsequent work. As indicated by the data in Table I, 2-propanol increases $\Delta\epsilon$ for the enzyme–dye complex and shifts the maximum slightly.

Table I. Binding of Biebrich Scarlet to α-Chymotrypsin[a]

% 2-propanol (v/v)	$10^5 K_D$, *M*	$\Delta\epsilon$ (λ, nm), M^{-1} cm^{-1}
0.0	2.26	12,365 (550)[b]
5.0	8.06	13,162 (553)
9.4	19.7	14,398 (555)

[a] Solutions buffered at pH 7 with 0.05 *M* phosphate at 25°. [b] Average of two determinations.

Data Analysis. The method of analysis used to obtain K_I and k_r depends upon a number of conditions and assumptions. These include: (1) the initial inhibitor concentration I_0 is in large excess over that of the enzyme, so that at any time $I \cong I_0$, (2) equilibria involving noncovalent complexes of the enzyme with either dye or inhibitor are achieved rapidly on a time scale relative to the reaction giving irreversibly inhibited enzyme, and (3) ternary complexes between chymotrypsin, Biebrich Scarlet, and inhibitor are not kinetically important.[26] The system may then be described as shown in eq 2–5, where D represents Biebrich Scarlet,

$$E + D \underset{}{\overset{K_D}{\rightleftharpoons}} ED \quad (2)$$

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k_r} \widehat{EI} \quad (3)$$

$$K_D = [E][D]/[ED] \quad (4)$$

$$K_I = [E][I]/[EI] \quad (5)$$

ED the enzyme–dye complex, and the other symbols have the same meaning given them in the introduction. By appropriate manipulation of eq 2–5, laws of mass balance and the assumptions outlined above, it is readily shown that

$$\frac{d[\widehat{EI}]}{dt} = k_r[EI] = k_r \frac{I_0 K_D[ED]}{K_I(D_0 - [ED])} \quad (6)$$

(20) A. M. Gold and D. Fahrney, *Biochemistry*, **3**, 783 (1964).
(21) A. N. Glazer, *J. Biol. Chem.*, **242**, 4528 (1967).
(22) J. Winkelman and S. S. Spicer, *J. Histochem. Cytochem.*, **11**, 489 (1963).
(23) G.-L. Rossi, E. Holler, S. Kumar, J. A. Rupley, and G. P. Hess, *Biochem. Biophys. Res. Commun.*, **37**, 757 (1969).
(24) R. Jayaram and I. D. Rattee, *Trans. Faraday Soc.*, **67**, 884 (1971).
(25) F. J. Kezdy and E. T. Kaiser, *Methods Enzymol.*, **19**, 1 (1970).
(26) That such a ternary complex may exist has been suggested for chymotrypsin, proflavine, and tosylarginine methyl ester. Although this complex was labile and not detectable under ordinary equilibrium conditions, it was kinetically significant. The time scale used in the temperature-jump experiment which indicated the ternary complex was much shorter than for our work: *cf.* G. H. Czerlinski and C. Odell, *Can. J. Biochem.*, **50**, 257 (1972).

where D_0 is the total (initial) dye concentration. Since it may also be shown that

$$\frac{d[\widehat{EI}]}{dt} = -\frac{d[ED]}{dt}\left\{1 + \frac{\alpha}{D_0 - [ED]} + \frac{\alpha[ED]}{(D_0 - [ED])^2}\right\} \quad (7)$$

where $\alpha = K_D(1 + I_0/K_I)$, it follows that

$$\frac{-d[ED]}{dt} = \left\{\frac{k_r(\alpha - K_D)(D_0 - [ED])}{D_0^2 - 2[ED]D_0 + [ED]^2 + \alpha D_0}\right\}[ED] \quad (8)$$

Integration yields

$$t = Q\left\{\ln\left(\frac{D_0 - [ED]}{D_0 - ED_0}\right) + \ln\frac{ED_0}{[ED]}\right\} + R\left\{\ln\frac{ED_0}{[ED]} + \left(\frac{[ED] - ED_0}{D_0}\right)\right\} \quad (9)$$

where $Q = (1 + K_I/I_0)/k_r$, $R = K_I D_0/K_D k_r I_0$, and ED_0 is the concentration of enzyme–dye complex at time 0. Equation 9 has the form

$$t = Qx + Ry \quad (10)$$

It was found empirically that the quantities corresponding to both x and y vary nearly linearly with time so that it was not possible in practice to determine Q and R independently. To get around this problem we assumed that the proportionality between the quantities x and y remained constant over the kinetic run. Calling the proportionality constant $\bar{D}$, eq 10 becomes

$$x = t/(Q + R\bar{D}) \quad (11)$$

A computer program was prepared which accepted the observed values of ED and time, calculated the quantities corresponding to x, y, and the value of $\bar{D}$ from these data, and performed a least-squares fit to eq 11. The parameter $(Q + R\bar{D})^{-1}$ gives k_r directly if K_I, K_D, and I_0 are known.

When the inhibitor I competes effectively with the dye for the enzyme binding site and the equilibria in eq 2 and 3 are rapidly established, the amount of enzyme–dye complex present immediately after mixing the three reagents will be less than that observed when the inhibitor is absent. It can be shown that

$$K_I = I_0\beta/(E_0 - ED_0 - \beta) \quad (12)$$

where $\beta = K_D ED_0/(D_0 - ED_0)$ and E_0 is the total enzyme concentration. Thus, from the initial value of the enzyme–dye concentration one can estimate K_I. As an example, the results of application of the above analysis to the reaction of 3-fluorobenzenesulfonyl fluoride with CT are given in Table II. The values of K_I and k_r obtained lead to a calculated optical density–time curve in good agreement with experiment (Figure 1). In this and other systems the derived values of K_I and k_r were found to be independent of the initial concentrations of dye and inhibitor; these observations give us confidence that the assumptions and procedures used in the analysis are valid.[27]

(27) In principle, determination of K_I and k_r can also be done by experiments in which I_0 and D_0 are varied. For most of the inhibitors used in this work, solubility limitations precluded wide changes in the concentration of the inhibitor while the usable concentration range for the dye was limited by the constraints that observable amounts of complex must be formed but that the dye cannot be present in such a large excess that it competes too well with the inhibitor.

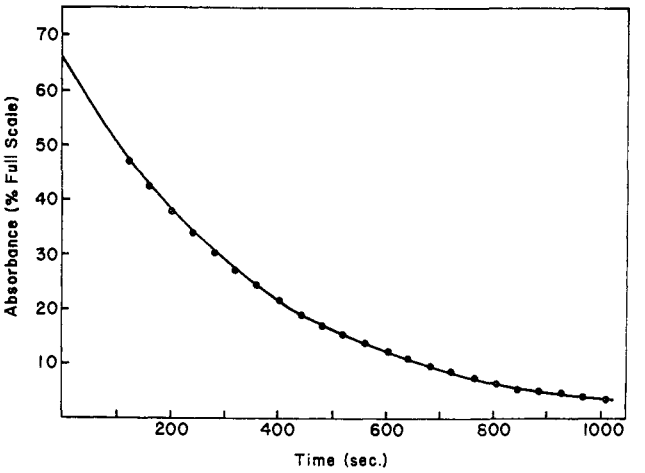

Figure 1. Observed differential absorbance at 553 nm for the chymotrypsin–dye complex in the presence of *m*-fluorobenzenesulfonyl fluoride as a function of time after mixing (points). The solid curve was obtained by Runge–Kutta integration of eq 8 using the values of K_I and k_r for this inhibitor given in Table II. 100% full scale was set at 0.200 absorbance unit; in this experiment the amount of complex expected at zero time in the absence of inhibitor corresponded to 124% of full scale.

Table II. Parameters Characterizing the Inactivation of α-Chymotrypsin by Substituted Benzenesulfonyl Fluorides[a]

Substituent	k_r, sec^{-1} × 10^3	K_I, mM	$k_r/K_I \times 10^{-1}$	$k_r/K_I \times 10^{-1}$, est.[c]
None	6.7	1.2	0.56	0.38
2-Fluoro[b]	3.4	1.3	0.26	0.21
3-Fluoro	5.9	0.6	0.98	0.58
4-Fluoro[b]	1.3	0.4	0.33	0.13
2,3,4,5,6-Pentafluoro[b]	48	1.6	3.0	3.1
4-Methyl	22	2.2	1.0	0.91
2-Trifluoromethyl	5.4	1.4	0.38	0.37
3-Trifluoromethyl				7.4
4-Trifluoromethyl[b]				3.3
3,5-Di(trifluoromethyl)				6.9
4-CF_3CH_2–				1.3
4-CH_3CF_2–[b]				2.1
4-$CF_3CH_2CH_2$–				0.73

[a] Run at pH 7.0 in 0.05 *M* phosphate buffer at 25°. Solutions contained 5% 2-propanol. Data quoted are averages of two or more separate determinations which always agreed to within 10%. Typically the concentrations of enzyme, dye, and inhibitor were 2–6 × 10^{-6}, 9 × 10^{-6}, and 0.4–1 × 10^{-3}, *M*, respectively. [b] Nonlinear semilog plots observed after *ca.* 2 half-lives. [c] Estimated according to the procedure given in the text.

It was found that, in most cases, the inhibitions were apparent first-order reactions in that they gave linear semilog plots of ED against time, often through 3 or more reaction half-lives. The slope of such a plot is called k_{obsd} below and provides an overall index of inhibitor reactivity. The observation that the disappearance of enzyme–dye complex is apparently first order suggests that the quantity inside the braces of eq 8 remains approximately constant throughout a kinetic run. This quantity can be equated to k_{obsd}. Some rearrangement shows that

$$k_{obsd} \approx \frac{k_r K_I I_0}{K_I D_0 + (K_I K_D D_0/(D_0 - [ED]) + K_D I_0 D_0/(D_0 - [ED]) - K_I[ED])} \quad (13)$$

Substitution of experimental values for the symbols within the parentheses of eq 13 indicates that, for the cases examined, this collection of terms does indeed remain rather constant as the reaction proceeds from the conditions $t = 0$, $ED = ED_0$ to $t = \infty$, $ED = 0$. Assuming the latter condition, one can derive

$$k_r/K_I \approx k_{obsd}(K_D + D_0)/K_D I_0 \qquad (14)$$

Table II gives the approximate values for k_r/K_I calculated by means of eq 14 for each of the inhibitors examined. Where comparison is possible these ratios agree moderately well with those obtained by the more accurate analysis outlined previously.

Kinetics. The dye displacement technique was applied to the study of 13 benzenesulfonyl fluoride inhibitors of CT. A 14th compound, 4-fluorosulfonyl-5′,5′,5′-trifluoropentylbenzene, was too insoluble even in 5% 2-propanol solution for use. For seven of these compounds the separate estimation of the K_D and k_r parameters was possible. These data are recorded in Table II. For the six remaining materials low solubility of the inhibitor (<0.3 mM) did not allow the first assumption listed above to be well satisfied and we could measure only k_{obsd}. This latter information was used to estimate k_r/K_I as indicated above.

With 2-fluoro-, 4-fluoro-, 4-trifluoromethyl-, and pentafluorobenzenesulfonyl fluorides significant deviations from linear semilog plots and corresponding nonconformance to eq 11 were observed after 2 or so reaction half-lives. In these cases values for the rate and binding constants were obtained by analyzing the linear portion of the data. Our observation that decreasing the initial inhibitor concentration shortens the time period during which linearity is maintained in these reactions supports the notion that the inhibitor concentration decreases faster than an be accounted for by single reaction with the enzyme, to the point that the assumption $I_0 \approx I$ is invalid in the latter stages of the kinetic run. We have considered two possible additional reactions of these sulfonyl fluorides namely (1) enzyme-catalyzed hydrolysis to give the corresponding sulfonic acids and (2) multiple reaction at several sites on the enzyme. It is known that CT can catalyze the hydrolysis of sulfonyl fluorides[28] but the second possibility is contrary to precedent.[14,20] Moreover, we were able to show that the modified enzyme formed by reaction of α-chymotrypsin with 4-fluorobenzenesulfonyl fluoride contains only one 4-fluorobenzenesulfonyl group. We, therefore, favor the former explanation of the deviations observed.

Discussion

The data presented above indicate that the displacement by irreversible inhibitors of Biebrich Scarlet from its complex with chymotrypsin provides a useful method for measuring both K_I and k_r in a single kinetic experiment. Although this method was not successful for all of our inhibitors, the failure was due to the lack of solubility of the inhibitors themselves and not to any shortcoming in the method. Proflavine has been widely used to study the reaction and binding of small molecules with this enzyme.[29-31] A number of precautions are frequently observed when working with proflavine, including daily preparation of solutions and protection of them from light. It has also been shown that proflavine forms complexes with some substrates of chymotrypsin.[30] While Biebrich Scarlet has spectral properties comparable to those of proflavine, its use has the additional advantages that solutions of it appear to be stable, and that, at least with our sulfonyl fluorides, no interaction of the inhibitors with the dye could be detected as judged by comparing the visible spectrum of the dye in the presence and absence of inhibitor. The aggregation of the former dye,[32] especially at relatively high ionic strengths, is a disadvantage. The low ionic strength employed in this study and the presence of cosolvent are thought to favor the monomeric form of the dye, and hence aggregation has been neglected in the present work.

The rate and binding constants reported in Table II are not readily interpreted, and presumably reflect specific interactions of each inhibitor with the enzyme. Enhanced binding of *m*- and *p*-fluoro derivatives of *N*-trifluoroacetylphenylalanine (compared to the unsubstituted compound) has been observed previously[9] and has been attributed to possible interactions with Ser-189 in the back of the binding pocket. Similar interactions may be responsible for the enhanced binding we observe for *m*- and *p*-fluorobenzenesulfonyl fluorides. The most striking observation regarding the binding constant data in Table II is the relative insensitivity of the enzyme to fluorine substituents on the benzene ring of the inhibitors and suggests that such substitution does not grossly perturb the interaction of these inhibitors with the enzyme. However, the reasons for the variations in reactivity of the fluoro-substituted benzenesulfonyl fluorides as reflected by changes in k_r are not obvious.

Placement of a *m*-trifluoromethyl group on the benzene ring of the inhibitor has a striking accelerative effect on the inhibition reaction. Both the 3-trifluoromethyl and 3,5-di(trifluoromethyl)-substituted inhibitors show this effect equally and, if one presumes K_I for both compounds is similar, it is likely that the effect is not electronic in nature but results from some severe juxtapositional interaction of the CF_3 function with the enzyme.

Alkyl and fluoroalkyl groups at the para position of the inhibitor cause a mild to substantial acceleration of the reaction with the enzyme, presuming that changes in the k_r/K_I ratio reflect primarily changes in k_r. These effects, again, are probably not electronic in origin since both methyl, an electron-donating group, and trifluoromethyl, an electron-withdrawing substituent, accelerate the reaction.

Experimental Section

α-Chymotrypsin from Worthington Biochemical Corp. (three times recrystallized, salt-free; Lot No. CDI 0LC and CDI 1IC) was used without further purification. These preparations were respectively 78 and 83% active, as determined by titration with 2-nitro-4-carboxyphenyl-*N*,*N*-dimethylcarbamate.[33] Biebrich Scarlet

(28) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 233 (1968).
(29) S. A. Bernhard, B. F. Lee, and Z. H. Tashjian, *J. Mol. Biol.*, **18**, 405 (1966).
(30) K. G. Brandt, A. Himoe, and G. P. Hess, *J. Biol. Chem.*, **242**, 3973 (1967).
(31) G. P. Hess, J. McConn, E. Ku, and G. McConkey, *Phil. Trans. Roy. Soc. London, Ser. B*, **257**, 89 (1970).
(32) D. N. Hague, J. S. Henshaw, V. A. John, M. J. Pooley, and P. B. Chock, *Nature (London)*, **229**, 190 (1971).
(33) B. F. Erlanger and F. Edel, *Biochemistry*, **3**, 346 (1964).

was obtained from Matheson Coleman and Bell, and was purified by the method of Jayaram and Rattee[24] with the modification that potassium acetate was used to salt out the dye. The Biebrich Scarlet thus precipitated was collected in small portions on a sintered glass filter, washed with boiling 95% ethanol, and dissolved in a minimum amount of water. Solutions used in further recrystallizations were made up to a convenient volume, and potassium acetate was again added. After five recrystallizations the Biebrich Scarlet solution was lyophilized; the resulting powder was dried in an Abderhalden pistol for 24 hr (0.3 mm, 60°) and stored in an actinic bottle. A portion of the purified dye was recrystallized three times more.

The extinction coefficient of the recrystallized Biebrich Scarlet was determined over the concentration range 0.5–5.0 × 10^{-5} *M*, and the absorbance of the dye at 505 nm adhered strictly to Beer's law. The concentration of Biebrich Scarlet in the spectrophotometric experiments was always kept below 10^{-4} *M* since, at higher concentrations, self-association of the dye can be shown.[21,32] Stock solutions of five times recrystallized Biebrich Scarlet (3 × 10^{-4} *M*) were made up in distilled, deionized water, and the dye concentrations were determined spectrophotometrically using the value ϵ 35,900 at 505 nm.

Proton nmr spectra were recorded on a Varian Associates T-60 spectrometer using *ca.* 5% tetramethylsilane (TMS) as an internal reference. Fluorine nmr spectra were obtained with a Varian Associates HA-100 spectrometer operating at 94.1 MHz. Infrared spectra were routinely taken by placing samples between salt plates and recording on a Perkin-Elmer 337 Grating Infrared spectrophotometer.

The extinction coefficient of the dye, the extinction coefficient difference, and dissociation constant of the enzyme–dye complex were determined from measurements performed with a Cary Model 15 recording spectrophotometer operating at ambient temperature. Kinetic experiments were performed with a Gilford 2000 spectrometer operating at the wavelength for which $\Delta\epsilon$ was a maximum (553 nm) in the presence of 5.0% 2-propanol. The thermostated sample cell holder was maintained at 25.0°.

Inhibitors. ***p*-Toluenesulfonyl fluoride** was obtained from Aldrich and was used directly.

Benzenesulfonyl Fluoride. The method of De Cat and Van Poucke[34] with a minor modification[35] was followed in the conversion of most sulfonyl chlorides to the corresponding sulfonyl fluorides. To 69 g (0.39 mol) of benzenesulfonyl chloride (Aldrich) in 100 ml of dioxane was added 40 ml of water containing 36.6 g (0.63 mol) of potassium fluoride. The mixture was refluxed for 2 days and the product distilled, bp 77–78° (3 mm) [lit.[36] bp 83° (3 mm)]. In this and all other cases the presence of the sulfonyl fluoride was confirmed by ir and fluorine nmr.[37] The absence of sulfonyl chloride was checked by testing with silver nitrate and ammonium thiocyanate.[38]

4-Fluorobenzenesulfonyl Fluoride. 4-Fluorobenzenesulfonyl chloride (5 g, 0.026 mol; Aldrich, mp 33–35°) was converted to the sulfonyl fluoride by the method of Sigler, *et al.*[39] The liquid product (0.015 mol) was distilled at 133–136° (*ca.* 15 mm) and was shown to be the desired product by the same methods used for benzenesulfonyl fluoride.

3-Fluorobenzenesulfonyl Fluoride. 3-Fluorobenzenesulfonyl chloride was prepared from 3-fluoroaniline by the method of Meerwein, *et al.*,[40] as modified by Yale and Sowinski.[41] To 11.1 g (0.1 mol) of 3-fluoroaniline in 35 ml of concentrated hydrochloric acid and 10 ml of glacial acetic acid at −5° was added dropwise 9.1 g (0.1 mol) of sodium nitrite in 18 ml of water. The solution was warmed to 4° and added to 3.3 g of cupric chloride dihydrate in 120 ml of a saturated solution of sulfur dioxide in acetic acid. After 30 min, the mixture was poured into 100 ml of ice–water and extracted with ether; the ether was washed with saturated sodium bicarbonate, dried over magnesium sulfate, and evaporated. The residue was submitted to the exchange reaction[39] and the crude sulfonyl fluoride was distilled (bp 26–33° (0.6 mm)).

2-Fluorobenzenesulfonyl Fluoride. Freshly distilled 2-fluoroaniline (Pierce) was converted to 2-fluorobenzenesulfonyl fluoride following the procedure described for the 3 isomer [bp 35–40° (0.2 mm)].

Pentafluorobenzenesulfonyl Fluoride. Pentafluorobenzenesulfonyl chloride (3.6 g, Aldrich) was submitted to the exchange reaction[34] and the sulfonyl fluoride distilled, bp 30–35° (0.3 mm).

4-Trifluoromethylbenzenesulfonyl Fluoride. 4-Aminobenzotrifluoride (Pierce) was distilled prior to use. Diazotization of the 4-aminobenzotrifluoride (4.2 g) was carried out as described for 3-fluoroaniline to yield 4-trifluoromethylbenzenesulfonyl chloride (bp 49.5–50.5° (0.2 mm), mp 29–31°, after two distillations). Conversion to the sulfonyl fluoride was accomplished after refluxing for several hours.[35] The product was sublimed at 40° (0.3 mm).

3-Trifluoromethylbenzenesulfonyl fluoride was prepared in a similar manner from 3-aminobenzotrifluoride (Aldrich). The product distilled at 52.5–53.0° (0.1 mm).

2-Trifluoromethylbenzenesulfonyl fluoride (15 mmol) was similarly obtained from 2-aminobenzotrifluoride (Aldrich). The product was distilled at 44.0–44.5° (0.3 mm) and was stored under nitrogen in a refrigerator since a previously prepared sample darkened when left overnight at room temperature.

3,5-Di(trifluoromethyl)benzenesulfonyl fluoride was prepared from 3,5-di(trifluoromethyl)aniline (Pierce) by a procedure similar to that used for the monotrifluoromethylbenzenesulfonyl fluorides. The product distilled at 33.0–35.5° (0.4 mm).

4-Fluorosulfonyl-1′,1′-difluoroethylbenzene. In a stainless steel bomb of 45-ml capacity was placed 5 g (0.022 mol) of 4-acetylbenzenesulfonic acid, sodium salt (Aldrich), along with a magnetic stirring bar and 0.4 ml of water. The bomb was cooled to acetone–Dry Ice temperature and evacuated to 0.1 mm. Sulfur tetrafluoride (16.5 ml, 0.276 mol; J. T. Baker) was distilled into the reaction vessel *via* a calibrated receiver on a vacuum manifold. The bomb was then sealed and heated at 110° for 48 hr. After the bomb was cooled to room temperature the gases were vented and the contents was poured into 30 ml of ice–water. The organic layer was taken up in dichloromethane, washed with sodium bicarbonate solution, and dried over magnesium sulfate. Evaporation of the solvent and two sublimations at 40° (0.3 mm) gave the desired compound as a white solid (0.016 mol) (mp 35–38°): ^{1}H nmr ($CDCl_3$) δ 1.95 (triplet, J = 18 Hz, 3 H), 7.80, and 8.11 (AB quartet, J = 8 Hz, 4 H).

4-Fluorosulfonyl-2′,2′,2′-trifluoroethylbenzene. Chlorosulfonic acid (80 ml, 1.22 mol; Eastman) was added slowly to a carbon tetrachloride solution of phenylacetic acid (36.2 g, 0.27 mol; Aldrich) containing 8.1 g (0.14 mol) of sodium chloride.[42] When the evolution of hydrogen chloride gas had diminished, the reaction mixture was warmed to 55° for 30 min and then cooled. The solution was poured over 1 l. of cracked ice and extracted with three portions of dichloromethane. The extracts were combined and dried over magnesium sulfate. Evaporation of the solvent gave 4-chlorosulfonylphenylacetic acid (0.14 mol) as a white solid. The crude sulfonyl chloride was submitted to the exchange reaction[39] and the sulfonyl fluoride obtained after recrystallization from dichloromethane (0.14 mol) was converted to the title compound using sulfur tetrafluoride by a procedure similar to that used for 4-fluorosulfonyl-1′,1′-difluoroethylbenzene. The crude 4-fluorosulfonyl-2′,2′,2′-trifluoroethylbenzene was purified by repeated sublimation (60° (0.15 mm)) to yield fine white crystals (mp 60–62°): pmr ($CDCl_3$) δ 3.55 (quartet, J = 10 Hz, 2 H), 7.64, and 8.07 (AB quartet, J = 8 Hz, 4 H).

4-Fluorosulfonyl-3′,3′,3′-trifluoro-*n*-propylbenzene. 4-Chlorosulfonylhydrocinnamic acid was obtained by chlorosulfonylation of hydrocinnamic acid (Aldrich), and the sulfonyl chloride was converted to the sulfonyl fluoride, by procedures similar to those just described. 4-Fluorosulfonylhydrocinnamic acid (0.02 mol) was heated (120°, 12 hr) in a bomb with sulfur tetrafluoride (0.15 mol) and titanium tetrafluoride as a catalyst (2.7 mmol). After normal work-up, the crude product was distilled (80.0–81.5° (0.3 mm)); the distillate solidified in the receiver. Sublimation (40° (0.2 mm)) afforded the desired product (9 mmol) (mp 38.5–39.0°): pmr ($CDCl_3$) δ 2.00–2.80 (complex multiplet, 2 H), 2.90–3.20 (multiplet, 2 H), 7.53, and 7.95 (AB quartet, J = 8 Hz, 4 H).

4-Fluorosulfonyl-5′,5′,5′-trifluoro-*n*-pentylbenzene (13 mmol) was obtained from 5-phenylvaleric acid (Aldrich) by a procedure similar

(34) A. De Cat and R. Van Poucke, *J. Org. Chem.*, **28**, 3426 (1963).
(35) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 672 (1968).
(36) C. G. Swain and C. B. Scott, *J. Amer. Chem. Soc.*, **75**, 246 (1953).
(37) N. S. Ham, A. N. Hambly, and R. H. Laby, *Aust. J. Chem.*, **13**, 443 (1960).
(38) E. H. Huntress and F. H. Carten, *J. Amer. Chem. Soc.*, **62**, 511 (1940).
(39) P. B. Sigler, B. A. Jefferey, B. W. Matthews, and D. M. Blow, *J. Mol. Biol.*, **15**, 175 (1966).
(40) H. Meerwein, G. Dittmar, R. Gollner, K. Hafner, F. Mensch, and O. Steinfort, *Chem. Ber.*, **90**, 841 (1957).
(41) H. L. Yale and F. Sowinski, *J. Org. Chem.*, **25**, 1824 (1960).
(42) E. E. Gilbert, "Sulfonation and Related Reactions," Interscience, New York, N. Y., 1965, p 84.

to that given above (bp 95–99° (0.3 mm)): pmr ($CDCl_3$) δ 1.3–1.8 (multiplet, 4 H), 1.8–2.4 (complex multiplet, 2 H), 2.5–3.0 (broad triplet, 2 H), 7.46, and 7.94 (AB quartet, J = 8 Hz, 4 H).

Titration of Biebrich Scarlet with Chymotrypsin. Stock chymotrypsin solutions were prepared by dissolving the enzyme (5–30 mg per ml) in 0.05 M phosphate buffer at pH 7.0. Varying amounts of such solutions were then promptly added to vials containing a fixed amount of dye (*e.g.*, 200 μl of 3.70×10^{-4} M Biebrich Scarlet) and an amount of buffer to bring each sample to an appropriate volume. The range of enzyme concentration was $0.1–5.0 \times 10^{-4}$ M after correcting for the percentage of active sites. Spectrophotometric experiments were always begun within an hour after the solutions were prepared, the time being allowed for the samples to come to thermal equilibrium. Difference spectra in the range of 650–450 nm were recorded by comparing the absorption of Biebrich Scarlet in the chymotrypsin solutions with that in the reference solution containing no protein. The same experiments were repeated for 0.05 M phosphate buffer containing various amounts of 2-propanol.

Kinetics. Kinetic experiments were initiated by adding 100 μl of a concentrated solution (10–40 mM) of sulfonyl fluoride inhibitor in 2-propanol to 3.0 ml of *ca.* 0.05 M phosphate buffer (pH 7.0) containing 1.83% 2-propanol, chymotrypsin ($2–6 \times 10^{-5}$ M), and Biebrich Scarlet (9.0×10^{-5} M). The final concentration of 2-propanol was 5.0%. Rates were determined by following decreasing absorbance at the wavelength of maximum $\Delta\epsilon$ (553 nm). Since 2-propanol perturbs the binding of the dye to the enzyme, a control containing only this cosolvent was also run in each case, and the decreasing absorbance was corrected for this perturbation. An attempt was made to maintain the inhibitor concentration in large excess of that of the enzyme, but due to the limited solubility of the inhibitors even in 5% 2-propanol, this excess was only 5–20-fold.

Reaction Stoichiometry. 4-Fluorobenzenesulfonylchymotrypsin was formed under conditions essentially identical with those used for the kinetic experiments using a fivefold molar excess of the irreversible inhibitor. 4-Fluorobenzenesulfonic acid was eliminated from a 0.88-g sample of this protein by the procedure of Weiner, *et al.*[16] A known amount of 4-fluorobenzoic acid was added to the reaction mixture and the solution concentrated. The fluorine-19 spectrum of the concentrate was recorded in the region 30–32 ppm upfield from external trifluoroacetic acid and, by using the relative peak areas of the two trifluoromethyl-substituted compounds, it was determined that 1.0 ± 0.05 mol of inhibitor was bound per mol of enzyme.

Acknowledgments. This work was supported by Grant CA-11220 from the National Cancer Institute and will constitute part of the doctoral thesis of D. C. R. The investigation was also supported by a U. S. Public Health Service Research Career Development Award (GM-70373) to J. T. G.

Inhibition of Chymotrypsin A_α with N-Acyl- and N-Peptidyl-2-phenylethylamines. Subsite Binding Free Energies[1]

James C. Powers,* B. Lee Baker, Janice Brown, and Barry K. Chelm

Contribution from the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332. *Received April 11, 1973*

Abstract: The dissociation constants for complexes of chymotrypsin A_α with a series of N-acyl- and N-peptidyl-2-phenylethylamine competitive inhibitors were measured in a 9.5% ethanol, 0.10 M $CaCl_2$ solution at pH 7.80 at 25°. Individual subsite binding free energies for the extended substrate binding site in chymotrypsin were calculated. The free energies of binding in this series of N-acyl- and N-peptidyl-2-phenylethylamines become more negative with increasing number of interactions with enzyme as predicted from the crystallographic model. The major contributors to the binding free energy are hydrophobic interactions, the S_1–P_1 tosyl pocket–phenylethyl group interaction (2 kcal/mol) and the interaction of an aliphatic side chain of a P_2 residue with Ile-99 in the S_2 binding subsite of the enzyme. The individual subsite binding free energies are not additive. Comparison of the dissociation constants of N-acyl- and N-peptidyl-2-phenylethylamines with the K_M values for the corresponding substrates indicates that the inhibitors are more tightly bound. This is consistent with the hypothesis that serine proteases place the scissionable peptide bond of a substrate on a stereoelectronic "rack" favoring formation of a tetrahedral intermediate.

Recent X-ray studies on chymotrypsin A_γ crystals inhibited with peptide chloromethyl ketone inhibitors (**1**) have shown the existence of an extended substrate binding site in this enzyme.[2] These active site directed irreversible inhibitors are bound to the enzyme *via* a covalent linkage between the imidazole ring of His-57 and the methylene group of the inhibitor (**2**). The benzyl group of the inhibitor occupies the so-called "tosyl pocket," a slit in the surface of the enzyme near the catalytic residues (Asp-105, His-57, and Ser-195)[3] and the peptide chain of the inhibitor forms an extended antiparallel β-sheet hydrogen bonding structure with the peptide backbone of residues Ser-214, Trp-215, and Gly-216 of the enzyme. Subsequent solution studies confirmed the relevance of the crystallographic results to the behavior of the enzyme in solution. Segal[4] showed that the K_M values derived from the rates of hydrolysis of seven peptide esters by chymotrypsin A_α were in accord with the crystal binding scheme. In addition, the rates of inhibition of chymotrypsin A_α with peptide chloromethyl ketones in solution were shown to correlate positively with ob-

(1) This investigation was supported by U. S. Public Health Service Grant No. GM-18292 and by the Research Corporation. A National Science Foundation undergraduate summer research fellowship (1972) is gratefully acknowledged (B. K. C.).

(2) D. M. Segal, G. H. Cohen, D. R. Davies, J. C. Powers, and P. E. Wilcox, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 85 (1971); D. M. Segal, J. C. Powers, G. H. Cohen, D. R. Davies, and P. E. Wilcox, *Biochemistry*, **10**, 3728 (1971).

(3) D. M. Blow, *Enzymes, 3rd Ed.*, **3**, 185 (1971).

(4) D. M. Segal, *Biochemistry*, **11**, 349 (1972).