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Inhibition Kinetics of Acetylcholinesterase with Fluoromethyl Ketones†

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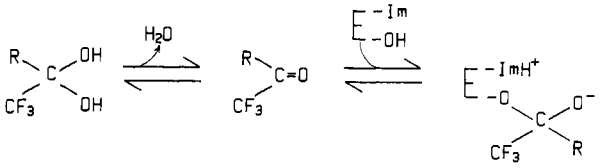
ABSTRACT: A series of trifluoromethyl ketones that reversibly inhibit acetylcholinesterase and pseudocholinesterase were synthesized. By analogy to chymotrypsin and on the basis of data reported here, we propose that the active-site serine adds to the ketone to form an ionized hemiketal. The compound (5,5,5-trifluoro-4-oxopentyl)trimethylammonium bicarbonate (**1**) inhibits acetylcholinesterase with K_i = 0.06×10^{-9} M and pseudocholinesterase with $K_i = 70 \times 10^{-9}$ M. Replacement of the nitrogen of **1** by carbon (compound **2**) increases K_i for **1** 200-fold for acetylcholinesterase but does not significantly alter K_i for pseudocholinesterase. The K_i for the methyl ketone corresponding to **2** is 2×10^{-4} M for both enzymes, as compared with 12×10^{-9} M for the trifluoromethyl ketone (acetylcholinesterase). For both enzymes, a linear decrease in log K_i with decreasing pK of the inhibitor hydrate was observed with ketones containing from 0 to 3 fluorines. We attribute this effect to the stabilization of the hemiketal oxyanion. The reduction of the pK of the hemiketal by the trifluoromethyl group is an important contributing factor to the low K_i of trifluoromethyl ketones. The inhibition of acetylcholinesterase by tetramethylammonium chloride and trifluoroacetone was compared to the inhibition by **1**, which is a composite of the two smaller inhibitors. The entropic advantage of combining the smaller inhibitors into one molecule is 1.1×10^3 M. Inhibitors with $K_i \leq 70 \times 10^{-9}$ M are slow binding (Morrison, 1982; Morrison & Walsh, 1988). The kinetic data do not require formation of a noncovalent complex prior to formation of the ketal, although such a complex(es) cannot be excluded. The hydrate/ketone ratios for trifluoro ketones as well as the rates of hydration and dehydration were measured with an $NADP^+$-dependent alcohol dehydrogenase and were found to be ~500, 0.25 s^{-1}, and 5×10^{-4} s^{-1}, respectively. The ketone, not the hydrate, reacts with the enzyme to form the enzyme–inhibitor complex. For "slow-binding" inhibitors, k_{on}, based on actual ketone concentration, ranges from 10^6 to 10^8 M^{-1} s^{-1}.

Trifluoromethyl ketones are potent inhibitors of acetylcholinesterase (EC 3.1.1.7) (Brodbeck, 1979; Gelb et al., 1987). These compounds also inhibit serine proteases. Trifluoromethyl ketones react with the active-site serine of chymotrypsin and elastase to form an ionized hemiketal as shown in Scheme I (K. D. Brady, D. Ringe, and R. H. Abeles, manuscript in preparation; Takahashi et al., 1988; Liang & Abeles, 1987). The pK of the hemiketal is <4.5 and that of the histidine is >10.5 (T.-C. Liang, K. D. Brady, and R. H. Abeles, manuscript in preparation). It is generally believed that the active-site structure and mechanism of catalysis of the serine proteases and the cholinesterases are similar (Hess, 1971). Inactivation of acetylcholinesterase by the fluorophosphate pinacolyl methylphosphonofluoridate reveals phosphorylation of a single serine on a unique polypeptide sequence, similar to that seen for chymotrypsin (Schaffer et al., 1973). Furthermore, the enzymatic hydrolysis of acetylcholine shows a similar pH dependence to ester hydrolysis by chymotrypsin; i.e., k_{cat}/K_m versus pH gives a bell-shaped curve with apparent pK's of ~6.5 and 10.5 and a flat region from pH 7.0 to 9.0 while k_{cat} has an apparent pK of ~6.5 (Rosenberry, 1975). The mechanism for both enzymes has been proven to proceed via acyl-enzyme formation (Froede & Wilson, 1984; Bentley & Rittenberg, 1954). It is, therefore, likely that the complex formed with trifluoro ketones and acetylcholinesterase has a structure similar to that formed with serine proteases.

Scheme I: Formation of Enzyme–Inhibitor Complex



In order to explore the mechanism of inhibition of acetylcholinesterase by trifluoro ketones, we have synthesized a number of fluoromethyl compounds isosteric to acetylcholine

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and have varied the number and position of fluorine atoms. Pseudocholinesterase (EC 3.1.1.8), which catalyzes the hydrolysis of a similar structure, butyrylcholine, was also used in this study. Of particular interest were the effect of the number of fluorine atoms and the positive charge of the trimethylammonium group on K_i of these inhibitors, as well as the basis for the relatively slow association rates for the inhibitors with the enzyme. We also determined whether the keto form or the hydrate of the trifluoro ketone combines with the enzyme.

Materials and Methods

Enzymes and Materials. Acetylcholinesterase from electric eel (type V-S), pseudocholinesterase from horse serum (type IV-S), alcohol dehydrogenase, $NADP^+$ dependent, from *Thermanaerobium brockii*, and β-NADPH tetrasodium salt (type X) were purchased from Sigma Chemical Co. and used without further purification. The substrates acetylthiocholine iodide and acetylcholine chloride were purchased from Aldrich Chemical Co. The substrates methyl acetate and ethyl acetate were purchased from Fisher Scientific and purified by distillation. All other organic compounds used were reagent grade or better.

Synthetic Procedures. Pentyl acetate was synthesized from pentanol and acetyl chloride and purified by distillation. The substrate 3,3-dimethylbutyl acetate was prepared according to Hasan et al. (1980). The inhibitors 1,1,1-trifluorobutan-2-one, 1,1,1-trifluoropentan-2-one, and 1,1,1-trifluorooctan-2-one were prepared from the corresponding bromides and lithium trifluoroacetate by a Grignard reaction (Sykes et al., 1956).[1] 1-Bromo-4,4-dimethylpentene, a synthetic intermediate, was prepared according to Whitmore and Homeyer (1933). The inhibitors **2** and **7** were prepared according to Gelb et al. (1985). Compounds **1**, **3**, **5**, **6**, and **8** were synthesized as described below. The inhibitor **4** was a gift from Dr. Michael Gelb.

Synthesis of (5,5,5-Trifluoro-4-oxopentyl)trimethylammonium Bicarbonate (**1**). The compound α-(trifluoroacetyl)-γ-butyrolactone, 8.0 g (40 mmol), prepared as previously described (Archer & Perianayagam, 1979), was combined with 10 mL of acetic acid and 0.4 mL of concentrated HCl and refluxed for 6 h. The aqueous phase was extracted with 1 volume of ethyl ether, dried over $MgSO_4$, and distilled to give 3.5 g (25 mmol, 63% yield) of 1,1,1-trifluoro-5-hydroxypentan-2-one, bp 75–78 °C (38 mm). Note that the product is cyclized, so the reaction time for the next step was lengthened considerably.

1,1,1-Trifluoro-5-bromopentan-2-one was prepared (Cohen et al., 1976) from the alcohol with modifications as follows. The reaction time was 12 h. Petroleum ether was added to the reaction mixture and the resulting precipitate filtered off before the solvent was removed by distillation to give 1.7 g (8 mmol, 36% yield) of 1,1,1-trifluoro-5-bromopentan-2-one, a colorless liquid: ^1H NMR ($CDCl_3$) δ 3.31 (t, 2 H), 1.74 (m, 2 H), 2.58 (t, 2 H).

Into a solution of 1,1,1-trifluoro-5-bromopentan-2-one (0.5 g, 2.3 mmol) in 3 mL of 1,2-dimethoxyethane cooled to 0 °C was bubbled 1 g of trimethylamine. The resulting solution, from which a precipitate began to form, was stirred at 25 °C for 12 h and then filtered. The residue was washed with diethyl ether and dissolved in H_2O, and the bromide was exchanged for bicarbonate on Dowex 1-X8. The aqueous solution was lyophilized to obtain 0.2 g (0.7 mmol, 32% yield) of a white powder, (5,5,5-trifluoro-4-oxopentyl)trimethylammonium bicarbonate (**1**): ^1H NMR (D_2O) δ 3.46 (t, 2 H), 3.18 (s, 9 H), 2.05 (m, 2 H), 1.91 (t, 2 H); ^{13}C NMR (D_2O) δ 168.5 (s, CO_3), 126 (q, CF_3, J_{C-F} = 288.1 Hz), 95.5 (q, CO), 68.2 (s, CH_2), 55.5 [s, $(CH_3)_3N$], 33.6 (s, CH_2), 18.3 (s, CH_2).

A mass spectrum was obtained: M^+ 277 (hydrated ketone, bicarbonate salt) and M^+ 259 (nonhydrated ketone, bicarbonate salt).

Synthesis of 1,1,1,3,3-Pentafluoro-6,6-dimethylheptan-2-one (**3**). 1,1,1-Trifluoro-6,6-dimethylheptan-2-one (**2**) (24 mmol) was brominated by the acid-catalyzed procedure (McBee & Burton, 1952) to yield 3-bromo-1,1,1-trifluoro-6,6-dimethylheptan-2-one (12.3 mmol, 50% yield): ^1H NMR ($CDCl_3$) δ 4.48 (t, 1 H), 2.01 (m, 1 H), 1.91 (m, 1 H), 1.29 (td, 1 H), 1.04 (td, 1 H), 0.83 (s, 9 H).

3-Bromo-1,1,1-trifluoro-6,6-dimethylheptan-2-one, 3.4 g (12.3 mmol), was dissolved in 20 mL of acetonitrile with anhydrous sodium acetate (1.01 g, 12.3 mmol) and 18-crown-6 (0.325 g, 1.23 mmol) and then refluxed for 4 h; the solution was filtered and the solvent removed by rotary evaporation to afford 1.3 g (43% yield) of 2-acetoxy-1,1,1-trifluoro-6,6-dimethylheptan-3-one. The product was purified by flash silica chromatography in a 1:1 chloroform:hexane mixture (R_f 0.25). Note that the product isomerized: ^1H NMR ($CDCl_3$) δ 5.45 (q, 1 H), 2.55 (m, 2 H), 2.25 (s, 3 H), 1.53 (m, 2 H), 0.90 (s, 9 H); ^{13}C NMR ($CDCl_3$) δ 199.5 (s, CO), 169.5 (s, CH_3CO), 122.4 (q, CF_3, J_{C-F} = 295 Hz), 74.5 (q, CF_3CO), 36.2 (s, CH_2), 36.4 (s, CH_2), 31.2 [s, $C(CH_3)_3$], 30.1 [s, $(CH_3)_3$], 21.2 (s, CH_3).

To 2-acetoxy-1,1,1-trifluoro-6,6-dimethylheptan-3-one (1.2 g, 5.4 mmol) under anhydrous conditions was added 1.77 g (0.011 mol) of (diethylamino)sulfur trifluoride at 0 °C. The mixture was heated at 40 °C for 12 h, quenched on 10 mL of ice/H_2O mixture, extracted into diethyl ether, and dried over $MgSO_4$. Solvent was removed by rotary evaporation to yield 0.9 g (3.5 mmol, 64% yield) of 1,1,1,3,3-pentafluoro-6,6-dimethylhept-2-yl acetate: ^1H NMR ($CDCl_3$) δ 5.56 (m, 1 H), 2.21 (s, 3 H), 1.90 (m, 2 H), 1.4 (t, 2 H), 0.92 (s, 9 H).

The acetyl group was removed by treatment with base (Imperiali & Abeles, 1986) to yield 1,1,1,3,3-pentafluoro-6,6-dimethylheptan-2-ol, which was purified by flash silica chromatography in a 3:7 hexane:chloroform mixture (R_f 0.4): ^1H NMR ($CDCl_3$) δ 4.10 (m, 1 H), 2.0 (m, 2 H), 1.42 (t, 2 H), 0.898 (s, 9 H).

The 1,1,1,3,3-pentafluoro-6,6-dimethylheptan-2-ol was oxidized to the ketone (Dess & Martin, 1983), using the sodium thiosulfate workup procedure. The ether was removed by distillation, and the resulting oil was dissolved in 1 N NaOH, acidified to pH 4.0 with HCl, and extracted into chloroform to yield the pure product, 1,1,1,3,3-pentafluoro-6,6-dimethylheptan-2-one (**3**): ^1H NMR ($CDCl_3$) δ 1.36 (t, 2 H), 1.93 (m, 2 H), 0.90 (s, 9 H); ^{13}C NMR ($CDCl_3$) δ 129.8 (m, CF_3, CF_2), 99.5 (m, CO), 69.3 (s, CH_2), 37.0 [s, $C(CH_3)_3$], 32.1 [s, $(CH_3)_3$], 18.0 (s, CH_2); ^{19}F NMR ($CDCl_3$) δ −81.9 (t, 3 F), −42.5 (m, 2 F).

Synthesis of 1,1-Difluoro-6,6-dimethylheptan-2-one (**5**). This compound was synthesized from 1-bromo-4,4-dimethylpentane and difluoroacetic acid by a Grignard reaction (Sykes et al., 1956). The product was purified by vacuum distillation, bp 75–83 °C (44 mm). The material migrated as a single spot on analytical TLC: R_f 0.61 ($CHCl_3$), 0.31 (CCl_4), 0.38 (2% 2-propanol in hexane); ^1H NMR ($CDCl_3$) δ 5.66 (t, 1 H, J = 54 Hz), 2.62 (t, 2 H), 1.6 (m, 2 H), 1.18 (m, 2 H), 0.88 (s, 9 H); ^{13}C NMR ($CDCl_3$) δ 199.98 (t, CO,

[1] The fluoromethyl ketones may exist as hydrates, ketones, or mixtures of both. They are all named as the ketone.

J_{C-F} = 27.7 Hz), 109.88 (t, CF_2H, J_{C-F} = 251 Hz), 43.38 (s, CH_2), 36.82 (s, CH_2), 30.34 [s, $C(CH_3)_3$], 29.18 [s, $(CH_3)_3$], 17.65 (s, CH_2).

Synthesis of 1-Fluoro-6,6-dimethylheptan-2-one (**6**). 1-Bromo-4,4-dimethylpentane, 7.7 g (43 mmol), was used to generate the magnesium salt in the usual manner with diethyl ether as solvent. CO_2 gas was introduced into the vessel (not bubbled into solution) with vigorous stirring for 90 min. The reaction mixture was quenched with 30 g of ice and 7.5 mL of concentrated HCl, extracted into diethyl ether, and dried with $MgSO_4$. The solvent was removed by rotary evaporation, yielding 4 g (28 mmol, 67% yield) of 5,5-dimethylhexanoic acid as white crystals, mp 38–39 °C: ^1H NMR ($CDCl_3$) δ 2.3 (t, 2 H), 1.6 (m, 2 H), 1.2 (m, 2 H), 0.9 (s, 9 H).

To a solution of 1.5 g (10.4 mmol) of 5,5-dimethylhexanoic acid in 5 mL of methylene chloride was added $SOCl_2$ (1.24 g, 10.4 mmol), and the solution was refluxed for 40 min. $SOCl_2$ was removed by rotary evaporation. Methylene chloride was added to the remaining residue and then removed by rotary evaporation. This was repeated several times. The mixture was cooled to 0 °C and to it was added an ethereal solution of diazomethane (20 mmol) until the yellow color persisted. The reaction was allowed to proceed for 60 min after which time the solvent was removed with a stream of nitrogen gas. The product was purified by flash silica chromatography in a 1:9 ether:petroleum ether mixture (R_f 0.3) to yield 0.71 g (4.2 mmol) of pure 1-diazo-6,6-dimethylheptan-2-one: ^1H NMR ($CDCl_3$) δ 5.2 (s, 2 H), 2.3 (t, 2 H), 1.6 (m, 2 H), 1.3 (m, 2 H), 0.9 (s, 9 H).

To a solution of 1-diazo-6,6-dimethylheptan-2-one (0.71 g, 4.2 mmol) in 20 mL of ether was added an aqueous solution of 47–49% HBr (1.35 mL) dropwise over 20 min at 15 °C. The reaction was allowed to proceed for 2.5 h, the mixture was extracted with ether, and the extract was dried with $MgSO_4$ and subjected to rotary evaporation to remove solvent from the product 1-bromo-6,6-dimethylheptan-2-one, 0.61 g (2.77 mmol, 66% yield): ^1H NMR ($CDCl_3$) δ 3.83 (s, 2 H), 2.6 (t, 2 H), 1.6 (m, 2 H), 1.2 (m, 2 H), 0.9 (s, 9 H).

To a solution of 18-crown-6 ether (0.06 g, 0.226 mmol) and KF (0.217 g, 4.52 mmol) in 2.3 mL of CH_3CN which had been allowed to stir for 30 min was added 1-bromo-6,6-dimethylheptan-2-one (0.5 g, 2.26 mmol). The reaction mixture was refluxed 3 h and extracted into ether, and the ether layer was washed with water and then brine and filtered. The ether was removed by distillation, affording 0.07 g (0.04 mmol) of 1-fluoro-6,6-dimethylheptan-2-one (**6**). The material migrated as a single spot on analytical TLC: R_f 0.58 ($CHCl_3$), 0.20 (CCl_4), 0.24 (2% 2-propanol in hexane); ^1H NMR ($CDCl_3$) δ 4.78 (d, 2 H, J = 24 Hz), 2.48 (5, 2 H), 1.58 (m, 2 H), 1.18 (m, 2 H), 0.88 (s, 9 H); ^{13}C NMR ($CDCl_3$) δ 207 (d, CO, J_{C-F} = 27.7 Hz), 85.3 (d, CH_2F, J_{C-F} = 184.7 Hz), 43.9 (s, CH_2), 39.4 (s, CH_2), 30.7 [s, $C(CH_3)_3$], 29.5 [s, $(CH_3)_3$], 18.3 (s, CH_2).

Synthesis of (R,S)-1,1,1-Trifluoro-6,6-dimethylheptan-2-ol (**8**). 1,1,1-Trifluoro-6,6-dimethylheptan-2-one (**2**), 0.127 g (0.65 mmol), was added dropwise to a solution of 0.65 mmol of lithium aluminum hydride in 5 mL of anhydrous diethyl ether. The reaction was heated at reflux for 2 h and then quenched by the dropwise addition of H_2O. The mixture was poured over 40 mL of ice/H_2O and the water layer extracted into diethyl ether. The ether solution was dried over $MgSO_4$ and the product purified by silica chromatography in a 2:1 hexane:ethyl acetate mixture (R_f 0.53), yield 31%. The material migrated as a single spot on analytical TLC: R_f 0.53 ($CHCl_3$), 0.25 (CCl_4), 0.21 (2% 2-propanol in hexane); ^1H NMR ($CDCl_3$) δ 3.92 (m, 1 H), 1.63 (m, 2 H), 1.24 (m, 4 H), 0.91 (s, 9 H); ^{13}C NMR ($CDCl_3$) δ 124.8 (q, CF_3), 70.5 (q, COH), 44.2 (s, CH_2), 31.5 [s, $C(CH_3)_3$], 29.8 [s, $(CH_3)_3$], 20.7 (s, CH_2), 14.6 (s, CH_2).

Analytical Procedures. Acetylcholinesterase (2×10^{-9} M) and pseudocholinesterase (0.05 unit/mL) were assayed spectrophotometrically in 20 mM potassium phosphate buffer, pH 7.0, with 0.5 mM acetylthiocholine at 412 nm (Ellman et al., 1961). The acetylcholinesterase concentration was determined by comparison of the apparent k_{cat} to that previously determined (Froede & Wilson, 1971). A unit of pseudocholinesterase is defined as the amount of enzyme which will hydrolyze 1.0 μmol of acetylcholine/min at pH 7.0 and 25 °C. Alcohol dehydrogenase (0.02–1.8 units/mL) was assayed in 20 mM potassium phosphate buffer, pH 7.0, and 0.24 mM β-NADPH with trifluoroacetone (4.6 mM) or compound **2** (6.2 mM) by following the decrease in absorbance at 340 nm. A unit of alcohol dehydrogenase is defined as the amount of enzyme which will reduce 1 μmol of acetone/min at pH 7.0 and 25 °C in the presence of 0.24 mM NADPH. All spectrophotometric assays were performed on a Perkin-Elmer 559 UV/vis spectrophotometer using 1-cm quartz cells at 25 °C.

Alternately, acetylcholinesterase (20×10^{-9} M) and pseudocholinesterase (1 unit/mL) were assayed in 0.1 mM potassium phosphate buffer, pH 7.0–7.3, with various substrates (see Table II) by recording the amount of 10 mM NaOH added over time to maintain a constant pH. The titration was performed on a Radiometer 11 titrator. All assays were performed at 25 °C.

All substrates were dissolved in acetonitrile such that the final concentration of organic solvent in the assays was 5% v/v, with the exception of acetylcholine and acetylthiocholine which were dissolved in the assay buffer. All inhibitors but **1** (which is water soluble) were dissolved in acetonitrile (with the total organic solvent present being maintained at 5% v/v).

All NMR data were acquired on a Varian XL300, 300-MHz NMR spectrometer. The standard to which ^{19}F NMR signals were referenced was trifluoroacetic acid (−77 ppm). The ^{19}F NMR spectra were not proton-decoupled.

Kinetic Parameters. For inhibitors which were not slow binding, values for K_i were determined from Lineweaver–Burk plots using initial velocities. Inhibitor concentrations used were $0.5K_i$–$5K_i$. For slow-binding inhibitors, K_i values were determined from the final velocities of reaction progress curves of product formation versus time as described by Cha (1975). Product formation was determined spectrophotometrically, with acetylthiocholine iodide as substrate. The assays were initiated by the addition of acetylcholinesterase (2×10^{-9} M) or pseudocholinesterase (0.05 unit/mL). The concentration ranges for inhibitors **2** and **3** used were (20–200) $\times 10^{-9}$ M and (20–100) $\times 10^{-9}$ M, respectively, for acetylcholinesterase. The concentration ranges for inhibitors **1**, **2**, and **3** used were (0.8–8) $\times 10^{-6}$ M, (0.6–2) $\times 10^{-6}$ M, and (40–300) $\times 10^{-9}$ M for pseudocholinesterase. Values for k_{on} and k_{off} were also determined from the progress curves (Williams & Morrison, 1979; Cha, 1975).

Additionally, k_{on} and k_{off} were measured by incubating acetylcholinesterase (2×10^{-9} M) or pseudocholinesterase (0.05 unit/mL) with inhibitor (in ≥100-fold excess), diluting the enzyme into the assay mixture at various time points, and measuring the initial velocity. The disappearance of enzymic activity followed pseudo-first-order kinetics, giving a value of k_{obs}. The k_{on} and k_{off} values were obtained from plots of k_{obs} versus inhibitor concentration (from the expression $k_{obs} = k_{on}[I] + k_{off}$) (Morrison, 1982).

For inhibitors **2** and **3**, k_{off} was determined by dilution of the enzyme–inhibitor complex. The complex was isolated by incubating acetylcholinesterase (20×10^{-9} M) with 1×10^{-6} M inhibitor in a total volume of 0.1 mL for 30 min at 25 °C and passing the mixture through a 1-mL centrifugation column according to the method of Penefsky (1979) as previously modified (Allen & Abeles, 1989). The complex was diluted (1×10^{-10} M) into an assay solution with acetylthiocholine as substrate, and the progress curve thus obtained was fit to an exponential expression (Williams & Morrison, 1979; Morrison, 1982).

Inhibition of Acetylcholinesterase by Two Inhibitors. The inhibition of acetylcholinesterase by two inhibitors was analyzed for cooperative effects. Enzyme binding to two inhibitors can be envisioned as shown in eq 1a. Inhibition of the enzyme is described by eq 1b. In eq 1b, S is defined as substrate, P

$$\begin{array}{ccccccc} EB & \overset{K_B}{\rightleftharpoons} & E & \longrightarrow & ES & \longrightarrow & E + P \\ {\scriptstyle K_{BA}}\Big\| & & \Big\|{\scriptstyle K_A} & & & & \\ EAB & \overset{K_{AB}}{\rightleftharpoons} & EA & & & & \end{array} \quad (1a)$$

$$\frac{v_i}{v_o} = \frac{(1 + [S]/K_m)K_A}{[A] + ([A][B]M)/K_B + K_A([S]/K_m + [B]/K_B + 1)} \quad (1b)$$

as product, v_i is the inhibited velocity, and v_o is the velocity in the absence of inhibitor. Free enzyme, E, binds to either of two inhibitors, A or B, with dissociation constants K_A and K_B, or enzyme–inhibitor complex, EA or EB, binds to a second molecule of inhibitor with dissociation constants K_{AB} and K_{BA}. The value M is a constant which describes the relationship of K_B to K_{AB} as in eq 1c. Thus, when $M < 1$, negative cooperativity exists between two inhibitors; when $M > 1$, positive cooperativity exists; and when $M = 1$, there is no cooperativity. M was determined according to eq 1b by computer-fitting a plot of v_i/v_o versus [B] at a constant concentration of [A].

$$M = \frac{K_B}{K_{AB}} = \frac{[E][EAB]}{[EA][EB]} \quad (1c)$$

Determination of pK. The pK of compounds **2**, **3**, and **5** was determined from a plot of the ^{19}F NMR chemical shift versus pH. The inhibitors (1–5 mM) were dissolved in acetonitrile and placed in 20 mM phosphate buffer such that the final volume of organic solvent was 5%. The solution also contained 10% D_2O, and 1 mM trifluoroacetic acid as an internal standard (−77 ppm). The solution was adjusted with KOH to the appropriate pH, and the pH was checked again after acquisition. In all cases the pH was within 0.1 pH unit before and after acquisition. The ^{19}F NMR chemical shifts for **2**, **3**, and **5** were respectively −86.56 (singlet, pH 8.03), −81.95 (triplet, pH 7.21), and −136.49 (doublet, pH 8.82). The pK of compound **7** was previously determined (Bell, 1966). Compound **6** proved to be unstable at pH >9, so the pK was estimated from a plot of number of fluorines versus pK.

Kinetics of Hydration of Trifluoro Ketones. Alcohol dehydrogenase catalyzed the $NADP^+$-dependent reduction of trifluoro ketones. An initial burst was seen, followed by a steady-state rate of reduction. Assuming that the alcohol dehydrogenase reacts with the ketone form of the trifluoro compound, the amount of product formed during the initial burst was used to calculate the hydrate/ketone ratio (K_{hyd}) present at equilibrium. The alcohol dehydrogenase concentration used was large enough that a further increase in enzyme concentration did not alter the magnitude of the burst. The scheme for alcohol dehydrogenase catalyzed reduction of a compound with a ketone (I_k) and hydrate (I_h) form is depicted in eq 2a. The steady state velocity achieved is described by eq 2b. It is assumed that the amount of ketone is small

$$I_h \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I_k + E \xrightarrow{k_3} P \quad (2a)$$

$$\frac{1}{v} = \frac{K_{hyd}}{[E]k_3[I_t]} + \frac{1}{k_1[I_t]} \quad (2b)$$

compared to the amount of hydrate and that the total amount of trifluoro compound is not significantly depleted during the time of analysis. According to eq 2b, a plot of $1/v$ versus $1/[E]$ at a fixed trifluoro ketone concentration $[I_t]$ was constructed. The dehydration rate, k_1, was determined from the intercept, and the rate of hydration, k_2, was calculated from the relationship $K_{hyd} = I_h/I_k = k_2/k_1$.

Measurement of Proton Release. In experiments to measure proton release during inhibitor binding, the buffer components were removed from acetylcholinesterase by using a 17.5 × 0.9 cm Sephadex G-25 column in 0.2 M KCl adjusted to pH 9.0 with KOH. For each determination, a 1-mL solution of 0.2 M KCl, pH 9.0, was stirred under a slow stream of argon gas. Next, acetylcholinesterase (17×10^{-9} M) and compound **2** (3.2×10^{-4} M) were added and incubated for 20 min. The pH was determined before and after the 20-min incubation with inhibitor by using an Orian Research Model 701A Ionalyzer.

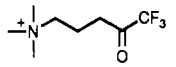
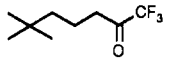
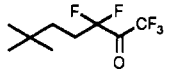
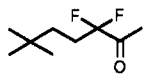
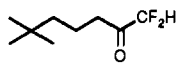
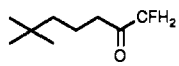
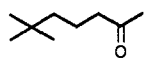
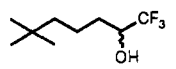
Results

Inhibition of Acetyl- and Pseudocholinesterase by Fluoro Ketones. The inhibitory effect of fluoro ketones on acetyl- and pseudocholinesterase was examined. The compounds used were selected to provide information concerning (1) the effect of the number of fluorine atoms on K_i and (2) the effect of the positive charge on K_i. The results are summarized in Table I. A comparison of K_i for compounds **1** and **2** shows that the presence of a positive charge is important for acetylcholinesterase, where it reduces K_i 200-fold, but not for pseudocholinesterase. This can be compared to the effect on the corresponding substrates. The presence of a positive charge increases V/K for acetylcholinesterase 200-fold (Cohen et al., 1984) while only a 16-fold increase is seen for pseudocholinesterase (compare the substrates acetylcholine and 3,3-dimethylbutyl acetate, Table II). An increase in the number of fluorine atoms, adjacent to the carbonyl group decreases K_i. This effect is seen for compounds containing up to three fluorine atoms. Introduction of two additional fluorine atoms (as in compound **3**) does not significantly change K_i. Compounds **4** and **5** contain two fluorine atoms, but in a different position relative to the carbonyl group. If the effect of fluorine were strictly electronic, both compounds should have the same K_i. This is the case for pseudocholinesterase. For acetylcholinesterase K_i for **4** is 80-fold lower than for **5**. An effect other than the inductive effect of fluorine must be invoked.

Figure 1 relates pK_i to the pK of the hydrate of the inhibitor. For acetylcholinesterase a linear relationship is observed between the pK of the hydrate and pK_i with a slope of −0.83. The pentafluoro compound deviates considerably, but also differs structurally, from the other inhibitors of the series. Similar results were obtained with pseudocholinesterase (data not shown; slope = −1.1). This experiment demonstrates that inhibitor binding increases with the ease of deprotonating the hydrate oxygen.

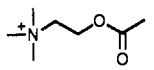
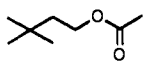
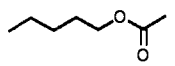
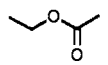
Association and Dissociation Rates of Trifluoro Ketones with Acetyl- and Pseudocholinesterase. Inhibitors with $K_i \leq 70 \times 10^{-9}$ M (compounds **1–3**, Table I) show slow-binding

Table I: Kinetic Constants for Fluoro Ketone Inhibitors of Cholinesterase

		K_i (M × 10^9)		k_{on} (M^{-1} s^{-1})[a]		k_{off} (s^{-1})	
		pseudo	acetyl	pseudo	acetyl	pseudo	acetyl
1		70[b]	0.06[b]	7×10^3 (3.6×10^6)	1.2×10^5 (6.2×10^7)	5×10^{-4}	6.7×10^{-6}
2		29[b]	12[b,c]	2.1×10^5 (1.1×10^8)	1.6×10^5 (8.3×10^7)	1.7×10^{-3}	2.5×10^{-3}
3		61[b]	11[b]	9.5×10^3 (4.9×10^6)[d]	3.1×10^4 (1.6×10^7)[d]	5.7×10^{-4}	2.5×10^{-4}
4		530	3[b,c]				
5		390	230				
6		4800	3700				
7		44 000	310 000[c]				
8		23 000[e]	170 000[e]				

[a] Values for k_{on} in parentheses have been corrected for the concentration of ketone. These are minimum values since they are calculated assuming the system is at equilibrium. [b] These inhibitors are slow binding (Morrison & Walsh, 1987). [c] Previously determined by Gelb et al. (1985). [d] This is a minimum value since the amount of ketone was taken as the amount present in equilibrium with trifluoroacetone. [e] The racemic mixture was used.

Table II: Kinetic Constants for Substrates and the Corresponding Inhibitors of Cholinesterase[a]

	K_i (M)[b]	K_m (M)	V/K (s^{-1}) × 10^7
	6.0×10^{-11} (7.0×10^{-8})	4.0×10^{-5} (1.6×10^{-4})	300 000 (88 200)
	1.2×10^{-8} (2.9×10^{-8})	6.7×10^{-3} (7.1×10^{-4})	1 400 (5 500)
	4.5×10^{-7}	1.9×10^{-2}	800
	1.5×10^{-4}	2.5×10^{-1}	3.8
	3.2×10^{-4}	2.0×10^{-4}	2.0

[a] Values are for acetylcholinesterase. Only those numbers in parentheses are for pseudocholinesterase. [b] K_i values are for the trifluoromethyl ketones corresponding to the given substrates.

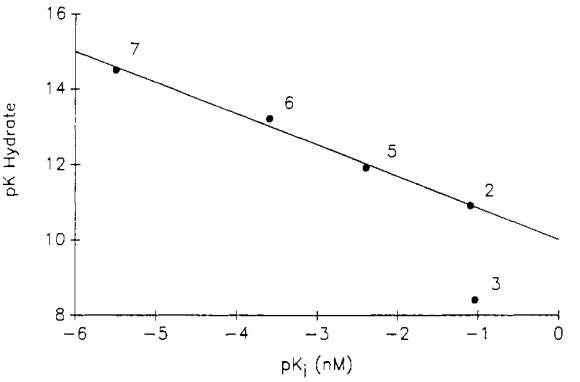


FIGURE 1: Effect of pK of inhibitor hydrate on inhibition of acetylcholinesterase. The pK's of compounds **2**, **3**, **5**, **6**, and **7** (points are numbered in the figure) were determined as described under Materials and Methods. The K_i values were determined as described under Materials and Methods.

kinetics (Morrison, 1982; Morrison & Walsh, 1988). This is evidenced by the decrease in the rate of product formation over time. Plots of k_{obs}, obtained from the progress curves, versus inhibitor concentration were used to calculate k_{on} and k_{off} for the slow-binding inhibitors with acetylcholinesterase and pseudocholinesterase. These plots are linear over the range of inhibitor concentrations used. The values for K_i, k_{on}, and k_{off} are summarized in Table I. In the case of compound **1**, K_i and k_{on} could not be determined from progress curves since the inhibitor concentration approached the enzyme concentration. The association rate was therefore determined by preincubating acetylcholinesterase with **1** and assaying over time for remaining activity. Enzymatic activity decreased in a first-order manner, giving a k_{on} of 1.2×10^5 M^{-1} s^{-1}. The value of K_i was calculated by using the equation for competitive inhibition which accounts for significant depletion of free inhibitor resulting from enzyme binding (Williams & Morrison, 1979). The association and dissociation rates (see Materials and Methods) for all other slow-binding inhibitors were also determined for pseudo-first-order kinetics. In all cases, the K_i values calculated from k_{on} and k_{off} determined by pseudo-first-order kinetics differ by less than 2-fold from those calculated from the equilibrium velocities of the progress curves.

In addition, k_{off} values for inhibitors **2** and **3** with acetylcholinesterase were directly determined by dilution of the isolated enzyme–inhibitor complex into the assay mixture such that the complex would completely dissociate (see Materials and Methods). From the rate of recovery of activity, k_{off} values of 1.2×10^{-3} and 3.0×10^{-4} were obtained for inhibitors **2** and **3**, respectively. These values are in good agreement with those obtained by using progress curves (see Table I).

Does the Enzyme Directly Interact with the Keto or Hydrate Form of the Inhibitors? In solution, trifluoro ketones are predominately present as the hydrate. The enzyme could react with the hydrate, catalyze its dehydration, and then form the tetrahedral adduct with the active-site serine. Alternatively, it could react directly with the keto form of the inhibitor to form the tetrahedral adduct. To approach this problem, we first attempted to obtain a quantitative evaluation of the

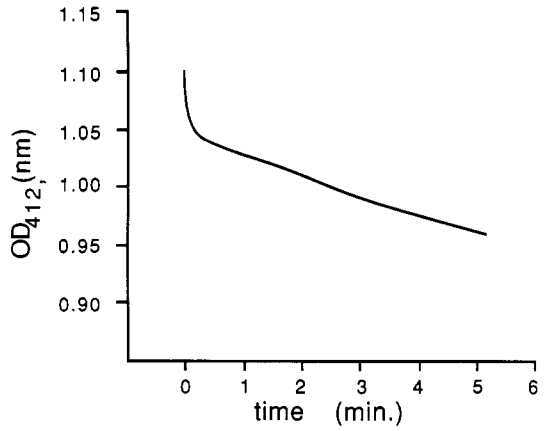


FIGURE 2: Reaction of alcohol dehydrogenase with trifluoroacetone. The reaction mixture contained 4.6×10^{-3} M trifluoroacetone, 0.02 unit/mL alcohol dehydrogenase, and 2.4×10^{-4} M NADPH in 20 mM potassium phosphate buffer, pH 7.0; total volume 0.4 mL. The disappearance of NADPH was measured by following the decrease in absorbance at 340 nm. Reactions were initiated by trifluoroacetone addition.

ratio of ketone to hydrate under the experimental conditions employed. We used trifluoroacetone in the initial studies. The $NADP^+$-dependent alcohol dehydrogenase (EC 1.1.1.2) catalyzes the reduction of trifluoroacetone. The kinetics of the reaction are shown in Figure 2. An initial burst is seen. It is reasonable to assume that alcohol dehydrogenase reacts only with the keto form of the substrate, and therefore, the initial burst represents the amount of keto form present. The kinetics of this system can be analyzed according to eq 2b (see Materials and Methods). Hydration (k_2) and dehydration (k_1) rates as well as the equilibrium constant (K_{hyd} = [hydrate]/[ketone]) were found to be 0.25 s^{-1}, 5×10^{-4} s^{-1}, and 502, respectively. The value for K_{hyd} for trifluoroacetone is in good agreement with the previously determined value of 396 measured by ^1H NMR (Smith et al., 1988). The equilibrium (K_{hyd}) constant obtained for inhibitor **2** was nearly identical.

The availability of an enzyme which reacts with the keto form of the inhibitor offers an opportunity to determine whether the keto form reacts directly with acetylcholinesterase. If the keto form of the inhibitor reacts with acetylcholinesterase, then the addition of alcohol dehydrogenase and NADPH should retard the formation of inhibited enzyme. If the hydrate reacts with cholinesterase to form a complex, then alcohol dehydrogenase will not retard complex formation, since the amount of inhibitor reduced by the alcohol dehydrogenase will not significantly reduce the concentration of the hydrate. It should be noted that the alcohol corresponding to inhibitor **2** is a poor inhibitor (see Table I, compound **8**). Acetylcholinesterase (2×10^{-9} M) and **2** (2×10^{-7} M) were allowed to react for 3 min and then were diluted into an assay mixture. The initial velocity obtained was 32% of the uninhibited rate. Another experiment was done under the same conditions except alcohol dehydrogenase (0.48 unit/mL) and NADPH (9.2×10^{-4} M) were added to the preincubation. The initial velocity obtained was 80% of the uninhibited velocity. Alcohol dehydrogenase reduces the rate of formation of enzyme–inhibitor complex. In separate experiments it was established that alcohol dehydrogenase or NADPH alone does not affect the rate of enzyme–inhibitor complex formation or the catalytic reaction. Furthermore, the reduction in the rate of inhibition by alcohol dehydrogenase and NADPH is not due to reduction of the total inhibitor concentration, since maximally 10% of the inhibitor was reduced during the 3-min incubation. This experiment establishes that the major pathway for formation of the enzyme–inhibitor complex is through reaction of the ketone with acetylcholinesterase.

Effect of pH on Inhibitor Binding. The K_i for acetylcholinesterase and compound **2** was determined at pH 9.0 (20 mM Tris-HCl buffer) by incubating enzyme with inhibitor for 60 min at pH 9.0, diluting the enzyme into the usual assay mixture at pH 7.0, and measuring the initial velocity. The K_i for acetylcholinesterase and **2** at pH 9.0 was identical with that at pH 7.0 ($K_i = 12 \times 10^{-9}$ M). The absence of a change in K_i argues against an ionizable group in the enzyme, inhibitor, or enzyme–inhibitor complex having a pK between pH 7.0 and 9.0.

Proton release upon enzyme–inhibitor complex formation was determined. Acetylcholinesterase (17×10^{-9} M) and inhibitor **2** were incubated in 200 mM KCl, pH 9.0 (see Materials and Methods). Under these conditions, the addition of 17×10^{-9} M HCl caused the pH of the solution to drop to 8.25. The pH of the solution was unchanged (9.00 ± 0.03) after incubation with the inhibitor. When an aliquot of this mixture was assayed for acetylcholinesterase activity, the enzyme was completely inhibited. This experiment further confirms that enzyme–inhibitor complex formation does not lead to proton release.

Relationship of K_i to V/K and K_s and the Connection Gibbs Energy. It has been pointed out that K_i for a series of transition-state analogues can be related to V/K for the corresponding substrates (Bartlett, 1983; Thompson, 1973). A plot of log K_i vs V/K for acetylcholinesterase is shown in Figure 3a. A linear relationship exists with slope −0.7 ($R = 0.98$). Figure 3b shows a plot of K_i vs K_s for acetylcholinesterase (see Figure legend for calculation of K_s). Here too a linear relationship is observed with slope 0.4 (but with a worse fit to the line, $R = 0.89$). The kinetic constants for the substrates and corresponding inhibitors are shown in Table II.

Inhibitor **1** minimally reacts with two regions of the active site: at the esteratic site through the carbonyl group and at the anionic site through the trimethylammonium group (Bergmann et al., 1950). Trifluoroacetone and tetramethylammonium chloride interact with the esteratic and the anionic site, respectively. Inhibitor **1** can be considered a composite of the two smaller inhibitors. We compared the inhibition by **1** to that obtained with tetramethylammonium chloride and trifluoroacetone to determine the advantage gained from incorporating the two components into a single molecule. Trifluoroacetone and tetramethylammonium chloride are competitive inhibitors of acetylcholinesterase with K_i values of 1.4×10^{-4} M and 4.8×10^{-4} M, respectively. The K_i values were determined from standard $1/v$ versus $1/S$ plots and are in agreement with published values (Brodbeck 1979; Wilson, 1952). From these values and the K_i for **1** of 0.06×10^{-9} M, the advantage from the connection Gibbs energy (Jencks, 1981) of 1.1×10^3 M can be calculated, i.e., $(4.8 \times 10^{-4}\ \text{M})(1.4 \times 10^{-4}\ \text{M})/(0.06 \times 10^{-9}\ \text{M})$. The observed advantage from connection Gibbs energy could be an entropic effect (Jencks, 1981) or a cooperative effect in that the occupation of the anionic site by tetramethylammonium ion could decrease K_i for trifluoroacetone at the esteratic site or vice versa. The inhibition of acetylcholinesterase by a combination of the two inhibitors was analyzed according to eq 1b (see Materials and Methods). A value of $M = 0.7 \pm 0.1$ was obtained, which indicates that there are no significant adverse steric or synergistic effects in the binding of these two inhibitors to acetylcholinesterase. The binding advantage gained from combining the two inhibitors into a single molecule appears to be entirely entropic. In order to establish the validity of this

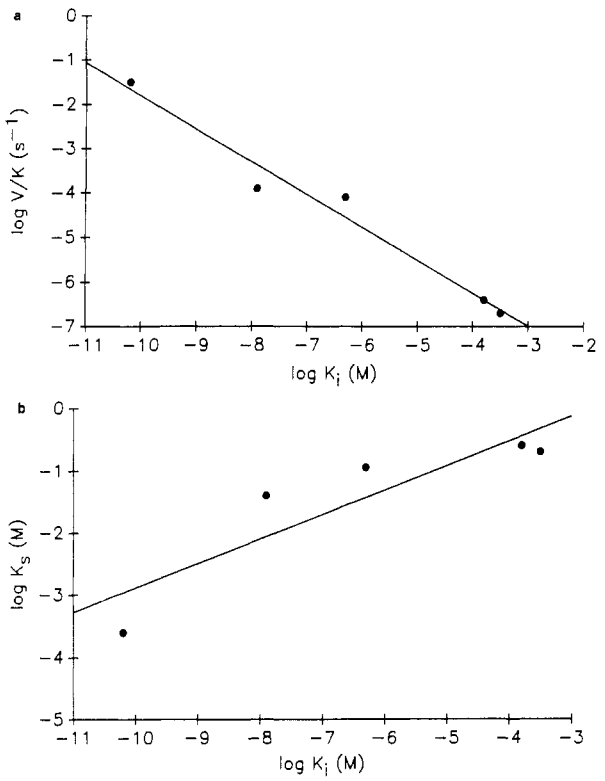


FIGURE 3: Relationship of K_i for inhibitors of acetylcholinesterase to V/K or K_s for corresponding substrates. The substrates used are listed in Table II. The substrates were assayed with acetylcholinesterase as described under Materials and Methods, and K_m and V_{max} values were determined from Lineweaver–Burk plots. Values for K_s were calculated from K_m values, since for good substrates $K_s \approx 6K_m$ and for poor substrates $K_s \approx K_m$ (Froede & Wilson, 1971). The inhibitors used were the trifluoromethyl ketones corresponding to the substrates in Table II. The first two compounds listed (**1** and **2**) were slow-binding inhibitors; all others were not. Values for K_i were determined as described under Materials and Methods. (a) Plot of log V/K versus log K_i. (b) Plot of log K_s versus log K_i.

experiment, two inhibitors were employed which are expected to show unfavorable steric interactions. The identical experiment was performed with the competitive inhibitor **7** (see Table I) and tetramethylammonium chloride. Analysis of the data by eq 1 gave $M = 0$, indicating that the two inhibitors cannot be bound at the active site simultaneously.

Discussion

Trifluoro ketones are effective inhibitors of acetylcholinesterase and pseudocholinesterase. For instance, compound **1** inhibits acetylcholinesterase with $K_i = 0.06 \times 10^{-9}$ M (Table I). We propose, by analogy with serine proteases, that these compounds react with the active-site serine to form an ionized hemiketal as shown in Scheme I. We have now demonstrated that formation of the enzyme–inhibitor complex does not require proton release, consistent with the mechanism in Scheme I. Furthermore, K_i is pH independent between pH 7.0 and 9.0 as has been observed for chymotrypsin–trifluoro ketone complexes (T.-C. Liang, K. D. Brady, and R. H. Abeles, manuscript in preparation). These results further confirm the mechanistic similarities of the two enzymes.

Fluorine substitution has a pronounced effect on K_i. Sequential introduction of fluorine atoms into the methyl group adjacent to the ketone decreases K_i. Comparison of inhibitors **2** and **7** for acetylcholinesterase shows that replacement of a methyl ketone by a trifluoromethyl ketone decreases K_i by a factor of 2.6×10^4. Information concerning the basis for the effect of fluorine on K_i is provided by the data in Figure 1. For both acetylcholinesterase and pseudocholinesterase, there is a linear correlation between pK of the inhibitor hydrate and pK_i of the inhibitor. These results are consistent with the formation of an enzyme–inhibitor complex as shown in Scheme I. Formation of that complex would be favored by a decrease in pK of the hemiketal. If the structure of the enzyme–inhibitor complex is similar to that of the chymotrypsin–inhibitor complex, then the oxyanion is stabilized by hydrogen-bond formation. While increasing the number of fluorines decreases pK, it also decreases the charge density available for hydrogen bonding. However, the stabilization of the oxyanion is the predominant effect; hence a decrease in pK of the hemiketal leads to a lower K_i.

It might be argued that the relationship of pK and pK_i does not pertain to oxyanion formation but instead reflects the increased electrophilicity of the carbonyl group. However, in forming the enzyme–inhibitor complex an OH group of the hydrate is replaced by a serine OH group (Scheme I). An increase in the electrophilicity of the carbonyl carbon would stabilize the hydrate to approximately the same extent as the serine–inhibitor adduct and would have no significant effect on K_i.

The ability of the inhibitor to simultaneously interact with several binding domains in the enzyme active site is important to the formation of enzyme–inhibitor complex. For instance, for trifluoroacetone $K_i = 1.4 \times 10^{-4}$ M. Elaboration of the structure of trifluoroacetone, by addition of a 3,3-dimethylbutyl group to inhibitor **2**, decreases K_i for acetylcholinesterase $\sim 10^3$-fold, and by addition of a 2-(trimethylamino)ethyl group to inhibitor **1**, decreases K_i 10^6-fold. We have evaluated the advantage gained from multisite interaction by comparing the inhibition by trifluoroacetone ($K_i = 1.4 \times 10^{-4}$ M) and tetramethylammonium ion ($K_i = 4.8 \times 10^{-4}$ M) to inhibition by inhibitor **1** ($K_i = 6 \times 10^{-11}$ M). Inhibitor **1** can be thought of as consisting of two components: tetramethylammonium ion and trifluoroacetone. Jencks (1981) has defined a term ΔG^s, the connection Gibbs energy. This represents the free energy contribution to binding from the entropic advantage of connecting two components of the inhibitor. The expression $RT \log [K_{AB}/(K_A)(K_B)] = 4.2$ kcal ($K_A = K_i$ for trifluoroacetone, $K_B = K_i$ for tetramethylammonium chloride, $K_{AB} = K_i$ for inhibitor **1**) approximates ΔG^s, since we have shown that the two inhibitors bind independently. The term $K_{AB}/(K_A)(K_B) = 1 \times 10^3$ M can be looked at in another way. Assume inhibitor **1** binds in two stages. If the tetramethylammonium group binds first with $K_i = 4.8 \times 10^{-4}$ M, then the binding constant for the trifluoroacetone component of **1** would be decreased 10^3-fold compared to the K_i of trifluoroacetone.

Inhibitors **1**–**3** are slow binding inhibitors as defined by Morrison (1982) and Morrison and Walsh (1988). The kinetic data are consistent with a reaction in which enzyme and inhibitor combine to form the final complex: $I + E \rightarrow EI$. No Michaelis-type noncovalent intermediate is required; i.e., k_{obs} did not show saturation with increasing inhibitor concentrations. Such an intermediate may occur, and very likely is formed, but is not detectable under the experimental conditions used. If the dissociation constant for the noncovalent complex is similar to K_s for substrates, it would be $\geq 2.4 \times 10^{-4}$ M (Froede & Wilson, 1971). The inhibitor concentrations used in these experiments were (0.02×10^{-6} to 2×10^{-6}) M; therefore, the intermediate complex cannot be detected. Since we have shown that the keto form of the inhibitor reacts with the enzyme, the on-rates can be recalculated on the basis of the concentration of the keto form. Corrected on-rates are diffusion controlled in the case of **2**, or near diffusion controlled

in the case of **1** and **3** (for corrected values see Table I). Other reactions (Morrison & Walsh, 1988) are known in which slow-binding inhibitors react at diffusion-controlled rates. A diffusion-controlled on-rate argues against the occurrence of a reversible enzyme–inhibitor complex prior to the formation of the tight binding complex, but does not exclude the prior formation of a committed complex. The rate of formation of the enzyme–inhibitor complex could be limited by the rate of ketone formation (i.e., the availability of inhibitor) when the enzyme concentrations is sufficiently high. For instance, at the highest enzyme concentration used ($\sim 2 \times 10^{-9}$ M), k_{on} for inhibitor **2** with acetylcholinesterase is probably limited by dehydration, i.e., $k_{dehydration} \approx k_{on}(\text{uncorrected})[E] \approx 5 \times 10^{-4}$ s^{-1}.

Does the enzyme catalyze the addition of the active-site serine to the ketone, and if so, how much is it accelerated compared to the nonenzymic addition of water? Several of the inhibitors described here form a complex with the enzyme at diffusion-controlled or nearly diffusion-controlled rates. That complex could be the covalent complex or a noncovalent complex which is eventually converted to the covalent complex. Whatever its structure, it is a highly committed complex. From a comparison of k_{on}(corrected), 1×10^8 M^{-1} s^{-1}, with the second-order rate constant for hydration, 0.0045 M^{-1} s^{-1} (0.25 s^{-1}/55 M), it can be determined that formation of the enzyme–inhibitor complex is 10^{10} faster than formation of the inhibitor–H_2O complex.

Are the fluoro ketones transition-state analogues? No unequivocal criteria exist to determine whether an inhibitor is a transition-state analogue. Supporting evidence is provided by a linear correlation between K_i and V/K (Bartlett & Marlowe, 1983; Thompson, 1973). Failure to obtain such a correlation does not exclude the possibility that an inhibitor is a transition-state analogue. We have observed a linear correlation between log K_i and log V/K as well as log K_i and log K_s. This suggests that interactions which stabilize the transition state may be involved in stabilizing the enzyme–inhibitor complex but are not solely responsible for inhibitor binding. The enzyme–inhibitor complex resembles the tetrahedral adduct which is believed to be an intermediate in the normal catalytic process. There is reason to believe that intermediate analogues have low K_i values (Schloss, 1988). The interactions which stabilize the tetrahedral intermediate in the catalytic process are probably very similar to those involved in the stabilization of the transition state since they are structurally very similar. It therefore becomes difficult, and perhaps meaningless, to attempt to define fluoro ketones as transition-state analogues or as intermediate analogues.

Acknowledgments

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