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Inactivation Studies of Acetylcholinesterase with Phenylmethylsulfonyl Fluoride

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ABSTRACT

Acetylcholinesterase (AChE), a serine hydrolase, is potentially susceptible to inactivation by phenylmethylsulfonyl fluoride (PMSF) and benzenesulfonyl fluoride (BSF). Although BSF inhibits both mouse and *Torpedo californica* AChE, PMSF does not react measurably with the *T. californica* enzyme. To understand the residue changes responsible for the change in reactivity, we studied the inactivation of wild-type *T. californica* and mouse AChE and mutants of both by BSF and PMSF both in the presence and absence of substrate. The enzymes investigated were wild-type mouse AChE, wild-type *T. californica* AChE, wild-type mouse butyrylcholinesterase, mouse Y330F, Y330A, F288L, and F290I, and the double mutant *T. californica* F288L/F290V (all mutants given *T. californica* numbering). Inactivation rate constants for *T. californica* AChE confirmed previous reports that this enzyme is not inactivated by PMSF. Wild-type mouse AChE and mouse mutants Y330F and Y330A all had similar inactivation rate constants with PMSF, implying that the difference between mouse and *T. californica* AChE at position 330 is not responsible for their differing PMSF sensitivities. In addition, butyrylcholinesterase and mouse AChE mutants F288L and F290I had increased rate constants (~14 fold) over those of wild-type mouse AChE, indicating that these residues may be responsible for the increased sensitivity to inactivation by PMSF of butyrylcholinesterase. The double mutant *T. californica* AChE F288L/F290V had a rate constant nearly identical with the rate constant for the F288L and F290I mouse mutant AChEs, representing an increase of ~4000-fold over the *T. californica* wild-type enzyme. It remains unclear why these two positions have more importance for *T. californica* AChE than for mouse AChE.

Acetylcholinesterase (AChE) is the enzyme that terminates the transmission of nerve impulses in cholinergic synapses by hydrolyzing the neurotransmitter acetylcholine to acetic acid and choline. AChE is a serine esterase with a catalytic mechanism resembling that of serine proteases such as trypsin. It possesses a high specific activity, functioning at a rate approaching that of a diffusion-controlled reaction (Voet and Voet, 1995). Inhibition of AChE is important both medically and toxicologically. Certain substances that covalently inhibit AChE are used as insecticides and as chemical warfare agents. Some inhibitors are used to treat various disorders such as myasthenia gravis and as a symptomatic approach to the management of Alzheimer's disease (Millard and Broomfield, 1995; Taylor, 1998).

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The X-ray crystal structure of *Torpedo californica* AChE was determined by Sussman et al. (1991). The structure revealed that the active site of *T. californica* AChE is located at the bottom of a narrow gorge approximately 20 Å deep and 4.4 Å in diameter at its narrowest. The active site gorge penetrates halfway into the enzyme and widens out close to its base. The van der Waals diameter of a tetramethylammonium ion is 6.4 Å, making this and other quaternary ammonium ions, such as acetylcholine (ACh) too large to enter the gorge (Axelsen et al., 1994). Substrates may gain access to the active site only if conformational changes occur with those residues that make up the gorge. The X-ray crystal structure of mouse AChE has also been determined by Bourne et al. and has the same general active site gorge structure as the *T. californica* enzyme (Bourne et al., 1999).

The active site contains a catalytic triad consisting of Ser200, His440, and Glu327. (Note: mammalian and *T. californica* AChEs have different numbering schemes. For consistency, the *T. californica* numbering scheme will be used throughout this article.) The triad is similar to that of trypsin and other serine

ABBREVIATIONS: AChE, acetylcholinesterase; ACh, acetylcholine; BChE, butyrylcholinesterase; PMSF, phenylmethylsulfonyl fluoride; BSF, benzenesulfonyl fluoride; ATC, acetylthiocholine chloride; FA, fractional activity.

proteases. However, the AChE triad has opposite stereochemistry to that of trypsin and contains Glu instead of the Asp that is in trypsin. The walls of the active site gorge are lined primarily by fourteen aromatic amino acid residues. Two of these residues (Trp84 and Phe330) interact with the quaternary ammonium ion of ACh (Axelsen et al., 1994).

In addition to AChE, most vertebrates have butyrylcholinesterase (BChE; an enzyme with a still-undetermined biological function), which hydrolyzes butyrylcholine in a manner similar to AChE hydrolysis of ACh. In fact, AChE is also able to hydrolyze butyrylcholine, although much more slowly than BChE, and BChE is able to hydrolyze ACh (Harel et al., 1992). Residues 4 to 534 of *T. californica* AChE can be aligned with residues 2 to 532 of mammalian BChE with more than 50% identity and no additions or deletions. In addition, the catalytic triad residues are in basically the same positions in both enzymes. Only 10 amino acids that have side chains facing the active site gorge differ between AChE and BChE. If the amino acid sequence of *T. californica* AChE and mammalian BChE are compared, there are two crucial residue changes (BChE residues are given in parentheses): Phe 288 (Leu) and Phe 290 (Ile or Val).

Both phenylmethylsulfonyl fluoride (PMSF) and benzene sulfonyl fluoride (BSF) are potential inhibitors of AChE. The sulfonyl group of PMSF and BSF mimics the carbonyl group of the ACh transition state (Fig. 1). The hydroxyl group of Ser200 nucleophilically attacks the sulfonyl group of PMSF or BSF, resulting in irreversible sulfonylation of AChE.

It has been reported that PMSF inactivates mouse AChE but does not react measurably with the enzyme from electric fish (eel or *T. californica*) (Fahrney and Gold, 1963; Barnett and Rosenberry, 1978; Moss and Fahrney, 1978). In contrast, BSF inhibits both mouse and fish AChE. PMSF has an extra methylene compared with BSF, and this extra carbon atom produces a profound change in inactivation of the fish enzyme. If the sequence of *T. californica* AChE is compared with that of mouse AChE, only five residues differ within the active site (mouse residues and numbering are given in parentheses): Glu 73 (Thr 75), Gln 74 (Leu 76), Ser 81 (Thr 83), Ser 124 (Ala 127), and Phe 330 (Tyr 337). The difference in reactivity may be caused by these few residue changes within the active sites or by the differing conformations of the active sites of the enzymes.

(−)-Huperzine A, a reversible inhibitor of AChE used in Chinese herbal medicines, has been shown to bind 25 times more tightly to mouse AChE than to the *T. californica* enzyme. However, the mouse Y330F mutant (*T. californica* numbering) has an affinity similar to that of the *T. californica* enzyme, indicating that the difference in amino acid at this position is an important contributor to the specificity for this drug (Saxena et al., 1994). We therefore undertook to examine the basis of the differing specificity of mouse and *T. californica* AChEs for PMSF, focusing first on the contribution of position 330. We also studied the effect of mutations at positions 288 and 290 on the PMSF specificity of the two enzymes. Positions 288 and 290 are located in the acyl pocket of the enzyme and are important in determining whether the enzyme is more specific for acetyl- or butyrylcholine. The specific enzymes we investigated were wild-type mouse AChE, wild-type *T. californica* AChE, wild-type mouse butyrylcholinesterase, and mouse Y330F, Y330A, F288L, and F290I (Radic et al., 1993; Vellom et al., 1993), and the double mutant *T. californica* F288L/F290V (Harel et al., 1992). Figure 2 shows the relationship of these residues at the active site with PMSF modeled into the site for orientation.

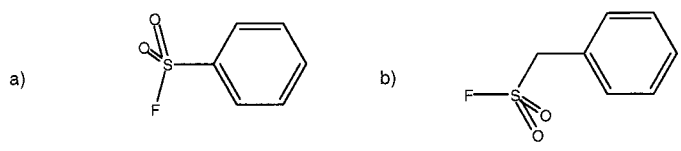


Fig. 1. Structures of BSF (a) and PMSF (b).

Materials and Methods

Chemicals. PMSF and BSF were purchased from Aldrich Chemical Company (Milwaukee, WI). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Chemical Company (Pittsburgh, PA).

Enzymes. The following enzymes were used: wild-type mouse AChE; four mutants of this wild-type enzyme (Y330A, Y330F, F288L, and F290I) (Radic et al., 1993); AChE from *T. californica*; wild-type mouse BChE; recombinant wild-type AChE from *T. californica*; and the double mutant F288L/F290V AChE from *T. californica* (Harel et al., 1992). AChE from *T. californica* was a kind gift of Drs. Jean Massoulié and Suzanne Bon (Laboratorie of Neurobiologie, Ecole Normale Supérieure, Paris, France).

AChE Assay. The assay was conducted as described in Ellman et al. (1961), using the following final reagent and enzyme concentrations: 0.5 mM acetylthiocholine chloride (ATC), 0.1 mM 2,2′-dithionitrobenzoic acid in 0.1 M potassium phosphate buffer, pH 7.4, and enzyme with an activity of about 0.1 ΔAbs_{412}/min/ml. The absorbance at 412 nm was measured for 1 min on a Cary Varian UV/Vis spectrophotometer. Units correspond to ΔAbs_{412}/min.

AChE Inactivation in the Absence of Substrate. The enzyme was diluted in 0.1 M phosphate buffer to an activity of approximately 2 ΔAbs_{412}/min/ml immediately before the experiment. Solutions of PMSF and BSF were prepared in methanol on the day of use. Inactivation reactions were carried out by adding 2.5 μL of inactivator solution to 0.5 ml of enzyme solution and incubating the mixture at 25°C. Aliquots (50 μl) were removed and diluted into assay medium (0.95 ml of assay medium in a 1.0 ml cuvette) every 2 to 4 min thereafter. Residual enzyme activity of each aliquot was measured by absorbance over 1 min at 412 nm.

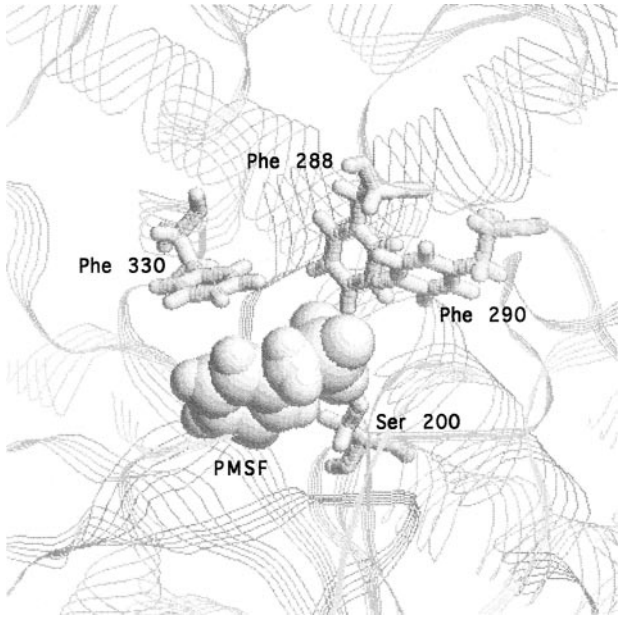
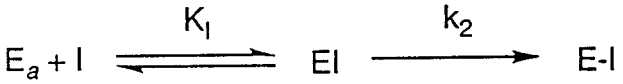


Fig. 2. The active site of *T. californica* AChE showing the active-site Ser(200) and the residues 288, 290, and 330. PMSF is modeled into the site for orientation.

Analysis of Data for AChE Inactivation in the Absence of Substrate. The mechanism of inactivation of AChE by BSF is assumed to involve the formation of a reversible enzyme-inhibitor complex followed by the covalent modification of the enzyme (Scheme 1).



Scheme 1

The fractional activity (FA) for this scheme is:

$$\ln \text{FA} = \ln\frac{v_t}{v_0} = -k't$$

where v_0 and v_t are the velocities of the enzymatic reaction at times 0 and t, and

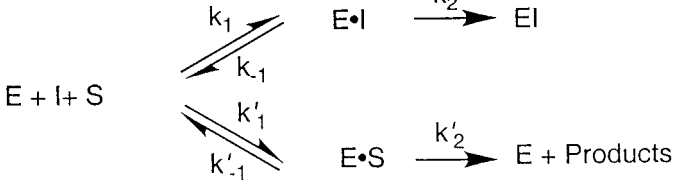
$$k' = \frac{k_2\,[\text{I}]}{K_\text{I} + [\text{I}]} \tag{1}$$

The natural logarithm of FA versus time was plotted and the value of k' determined.

Then by plotting k' versus [I], the values of k_2 and K_I were determined for the inactivation of the enzyme by a nonlinear least-squares fit of the data to eq. 1 using KaleidaGraph 3.0 (Synergy Software, Reading, PA). k_2/K_I is the second-order rate constant for the inactivation of free enzyme by inhibitor.

AChE Inactivation in the Presence of Substrate. The reaction mixture included 0.01 to 0.6 mM ATC and 1.0 mM 2,2′-dithionitrobenzoic acid in 0.1 M phosphate buffer, pH 7.4. Enzyme solution made with 0.1 M phosphate buffer, pH 7.4, was added to the reaction mixture to give an approximate activity of 0.005–0.05 ΔAbs/min/ml. PMSF and BSF solutions were freshly prepared in methanol with final concentrations in the reaction mixture ranging from 0.006 to 1.5 mM. The final methanol concentration was 0.1 to 0.5%. The reactions were carried out at 25°C. The absorbance at 412 nm was measured for 15 to 60 min using a Cary Varian UV/Vis spectrophotometer. K_m values for ATC were determined using ATC without inhibitor present.

Analysis of Data for AChE Inactivation in the Presence of Substrate. Inactivation assays were performed on mutant mouse and *T. californica* AChE using an in situ procedure for measuring the inactivation rates as described by Hart and O'Brien (1973). This procedure is based on using a system containing enzyme, inhibitor, and substrate. The general scheme for this system is as shown in Scheme 2, where I is the inhibitor, which in this case is PMSF or



Scheme 2

BSF. Kinetic analysis of the reactions gives the following equations (Hart and O'Brien, 1973)

$$\frac{1}{[\text{I}]} = \frac{k_2(1-\alpha)}{K_\text{I}} \cdot \frac{1}{k'} - \frac{1-\alpha}{K_\text{I}} \tag{2}$$

or

$$\frac{1}{(1-\alpha)} = \left(\frac{k_2}{K_\text{I}}\right)\left(\frac{[\text{I}]}{k'}\right) - \frac{[\text{I}]}{K_\text{I}} \tag{3}$$

where k_2 is the rate constant of sulfonylation of enzyme by PMSF, K_I is the dissociation constant of PMSF, k' is the pseudo–first-order rate constant for the disappearance of active enzyme, and α is given by the following equation:

$$\alpha = \frac{[\text{S}]}{[\text{S}] + K_\text{m}} \tag{4}$$

where [S] is the concentration of substrate and K_m is the Michaelis constant of the enzyme for ATC.

The values of k_2/K_I were obtained from a plot of 1/[I] versus $1/k'$ according to eq. 2 or from a plot of $1/(1-\alpha)$ versus $[\text{I}]/k'$ according to eq. 3.

k' was determined by one of two methods. Method 1: the derivatives of the graph of concentration of final product (thionitrobenzoate) with respect to time in both the presence and absence of inactivator were calculated. These derivatives are equal to the enzyme activity as a function of time, and yield FA. Plotting ln(FA) versus t yields k' values for each concentration of inhibitor, I. Method 2: integrating the FA equation

$$\ln \text{FA} = \ln\frac{v_t}{v_0} = \ln\frac{dA_{412}/dt}{v_0} = k't$$

gives the equation:

$$A_{412} = C - \frac{e^{\ln V_0 - k't}}{k'}$$

where C is a constant of integration. If the rate of nonenzymic hydrolysis of ATC (k_h) is taken into account, then the equation becomes

$$A_{412} = \left(C - \frac{e^{\ln V_0 - k't}}{k'}\right) + k_\text{h}[\text{ATC}]_t \tag{5}$$

A_{412} is plotted versus time and, using KaleidaGraph 3.0, a nonlinear regression to eq. 5 yields the value for k', with an R^2 typically greater than 0.99.

If substrate concentration is kept constant and inhibitor concentration is varied, a plot of 1/[I] versus $1/k'$ yields a straight line with a slope equal to $[k_2(1-\alpha)]/K_\text{I}$ and a y-intercept of $-(1-\alpha)/K_\text{I}$, as depicted in eq. 2. Similarly, if inhibitor concentration is kept constant but substrate concentration is varied, a plot of $1/(1-\alpha)$ versus $[\text{I}]/k'$ yields a straight line with slope k_2/K_I and intercept $-[\text{I}]/K_\text{I}$, as depicted in eq. 3.

The value of K_m for ATC was determined under the conditions described in the AChE assay above, but with [ATC] varying from 0.01 to 0.6 mM. The initial rate of substrate hydrolysis was plotted against [ATC], and the data were analyzed using a nonlinear curve fit to the Michaelis-Menten equation.

Using eq. 2 or eq. 3, k', and the measured value of K_m to determine α, it was possible to determine k_2 and K_I values and the second-order rate constant for inactivation (k_2/K_I).

Results and Discussion

The K_m values we obtained for each of the enzymes studied is shown in Table 1, along with their reported relative turnover numbers.

Figure 3 shows a representative plot for the inactivation of mouse AChE by 4 mM BSF or 0.5 mM PMSF in the absence of substrate. The reactions follow pseudo–first-order kinetics with rate constants, k', indicated on the graph. PMSF is the more reactive inactivator, as shown by the 8-fold higher BSF concentration necessary to achieve even a 6-fold slower inactivation than that using PMSF.

Figure 4 shows a representative time plot for the inactivation of *T. californica* AChE by 4 mM BSF or 4 mM PMSF in the absence of substrate. The time course for inactivation of the

enzyme in the presence of 0.5% methanol is shown for comparison.

As first reported by Fahrney and Gold (1963), Fig. 4 shows that BSF is clearly the more reactive inactivator of *T. californica* AChE. In fact, PMSF does not inactivate the *T. californica* enzyme significantly more than does 0.5% methanol, even under the extremely high concentrations used (4 mM).

The inactivation by BSF of *T. californica* AChE, mouse wild-type AChE, and several mouse mutants was examined in the absence of substrate. Figure 5 shows the plot of k' versus [BSF] for wild-type mouse AChE. This inactivation shows saturation kinetics and the data were analyzed using eq. 1 as described under *Materials and Methods*.

TABLE 1
Summary of K_m values for wild-type and mutant enzymes

Enzyme	K_m (mM)	Relative K_{cat}/K_m[a]
WT *T. californica* AChE	0.061 ± 0.004	1
WT mouse AChE	0.051 ± 0.008	1
Mouse Y330A	0.16 ± 0.02	0.15
Mouse Y330F	0.16 ± 0.02	0.43
WT mouse BChE	0.16 ± 0.03	
Mouse AChE F288L	0.052 ± 0.004	0.3
Mouse AChE F290I	0.19 ± 0.02	0.03
T. californica AChE F288L/F290V	0.101 ± 0.009	0.1

[a] With wild-type taken as 1; taken from references Harel et al. (1992) and Radic et al. (1993).
WT, wild-type.

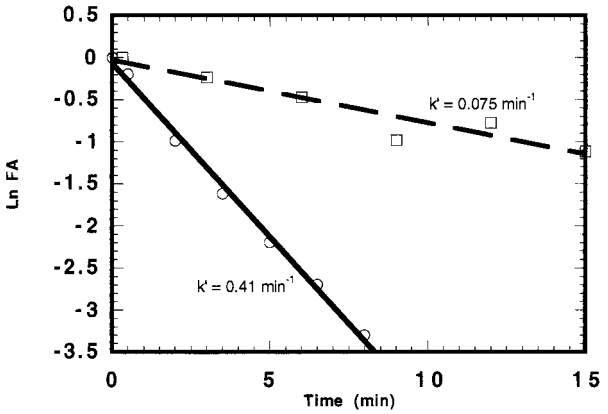


Fig. 3. Semilog plot for the FA of wild-type mouse AChE as a function of time in the presence of 4 mM BSF (□) or 0.5 mM PMSF (○).

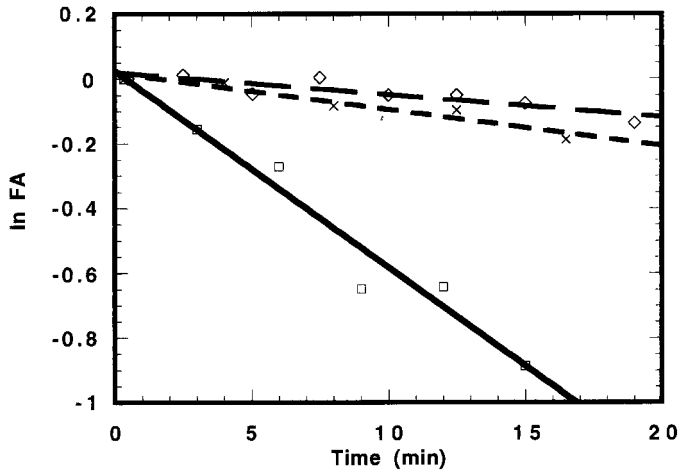


Fig. 4. Semilog plot for the FA of *T. californica* AChE as a function of time in the presence of 4 mM BSF (□) or 4 mM PMSF (◇). The inactivation of *T. californica* AChE by 0.5% methanol (×) is shown as a control.

BSF inactivation of AChE was also examined in the presence of substrate for wild-type *T. californica*, mouse, and both *T. californica* and mouse mutants, as will be described below. Table 2 gives values of k_2, K_I, and k_2/K_I for BSF inhibition in both the presence and absence of substrate. BSF reacts measurably with all enzymes assayed.

The two mutants assayed in the absence of substrate, mouse Y330F and mouse Y330A, have k_2/K_I values of 50 and 100 M^{-1} min^{-1}, respectively. Again, these values are not significantly different from the original mouse AChE k_2/K_I. These results confirm previous reports that BSF inhibits both mammalian and *T. californica* AChE (Barnett and Rosenberry, 1978) and show that mouse mutations at position 330 to the residues of either *T. californica* AChE (Y330F) or mouse BChE (Y330A) do not affect the inactivation significantly.

BSF inactivation of AChE in the presence of substrate (acetylthiocholine) was measured as follows: product formation was measured as a function of time for AChE in the presence of BSF and the resulting data were analyzed according to eq. 5 as described under *Materials and Methods* to obtain k' and k_2/K_I values, as shown in Fig. 6.

BSF inactivation in the presence of substrate gives k_2/K_I values with smaller associated error terms than for the absence of substrate assay for both wild-type *T. californica* and mouse, but in both cases, the two sets of data are consistent. Wild-type *T. californica* and mouse AChE have fairly slow rates of inactivation by BSF (k_2/K_I = 44 and 110 M^{-1} $min^{-}1$, respectively). Mutations in F288 and F290 have been shown to convert the acyl pocket of AChE to that of BChE (Harel et al., 1992; Vellom et al., 1993). All such acyl pocket mutations tested (Mouse F288L and F290I and *T. californica* F288I/F290V) greatly accelerate the rate of inactivation of the enzyme by BSF. Although the data are associated with larger errors for the individual rate and dissociation constants, it seems that at least some of the rate enhancement comes from better binding (K_I), whereas some may also come from an accelerated sulfonylation reaction (k_2), perhaps because of better BSF positioning in the mutant enzymes. Either the larger pocket can better accommodate the bulky benzene group or better position it to attack or the active site gorge as a whole is rendered more flexible, allowing BSF in more readily.

PMSF inactivation of various AChEs was studied both in

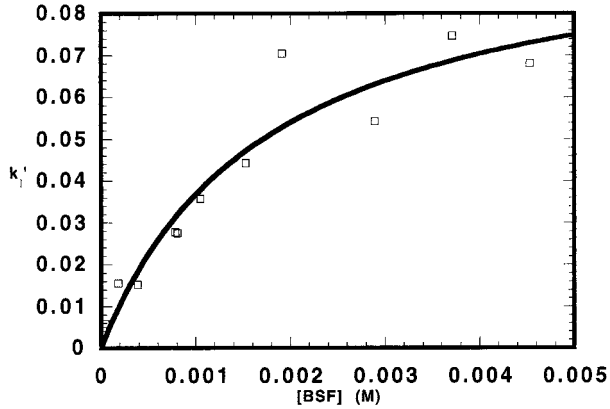


Fig. 5. The rate constant, k', for the inactivation of wild-type mouse AChE in the absence of substrate as a function of [BSF]. Data were analyzed as described under *Materials and Methods*.

TABLE 2
BSF inhibition of AChE mutants. Average of at least two independent experiments.

Enzyme	Absence of Substrate			Presence of Substrate		
	K_1 (mM)	k_2 (min^{-1})	k_2/K_1 (M^{-1} min^{-1})	K_1 (mM)	k_2 (min^{-1})	k_2/K_1 (M^{-1} min^{-1})
WT *T. californica*	4 ± 6	0.06 ± 0.05	14 ± 30	0.46 ± 0.08	0.020 ± 0.006	44 ± 6
WT mouse	2 ± 1	0.10 ± 0.02	60 ± 30	0.4 ± 0.2	0.036 ± 0.009	110 ± 20
Y330A mouse	3 ± 2	0.16 ± 0.05	50 ± 40	ND	ND	ND
Y330F mouse	0.9 ± 0.3	0.10 ± 0.01	100 ± 60	ND	ND	ND
F288L mouse	ND	ND	ND	0.04 ± 0.01	0.15 ± 0.03	4,400 ± 700
F290I mouse	ND	ND	ND	0.021 ± 0.004[a]	0.22 ± 0.05[a]	12,000 ± 1000
F288L/F290V *T. californica*	ND	ND	ND	0.12 ± 0.2[a]	0.18 ± 0.04[a]	1,200 ± 300

[a] Data from one experiment only used to determine this variable.
WT, wild-type; ND, not determined.

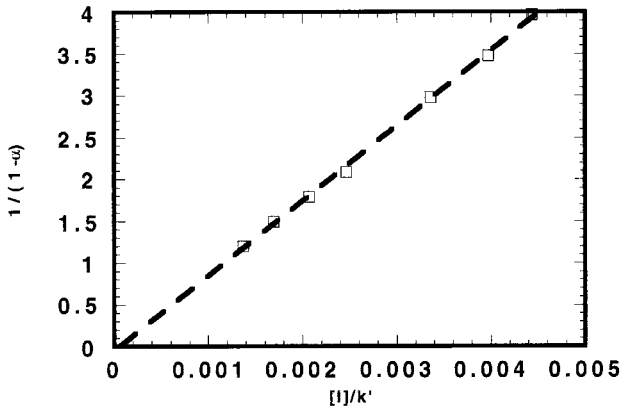


Fig. 6. Plot of $1/(1 - \alpha)$ versus $[I]/k'$ for the inactivation of *T. californica* F288L/F290I in the presence of 0.12 mM BSF, fit to eq. 3. The slope equals k_2/K_I as described under *Materials and Methods*.

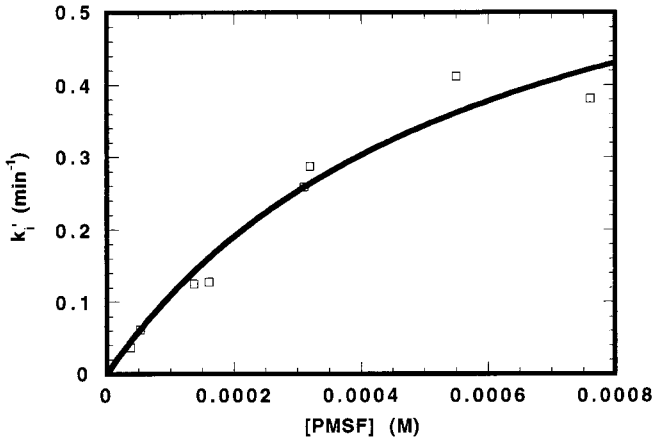


Fig. 7. The rate constant, k', for the inactivation of wild-type mouse AChE in the absence of substrate as a function of [PMSF]. The data were analyzed as described under *Materials and Methods*.

the absence and presence of substrate. The plot of k' versus [PMSF] for wild-type mouse AChE in the absence of substrate is shown in Fig. 7. Although the concentration of PMSF was not high enough to saturate the enzyme, the data could still be analyzed as above for BSF to obtain k_2 and K_I values. Data from inactivation in the presence of substrate were analyzed as for BSF as well.

The k_2/K_I values for the inactivation by PMSF of wild-type *T. californica* and mouse AChEs, mouse mutants Y330F, Y330A, F288L, and F290I, *T. californica* double mutant F288L/F290V, and mouse BChE are shown in Table 3. Each enzyme was assayed in both the absence and presence of substrate.

Comparison of the reactivities of BSF and PMSF in inactivating mouse AChE (Tables 2 and 3) shows that BSF second-order inactivation constants for mouse AChEs are 10-fold less than PMSF second-order inactivation constants for the same enzyme. Despite the increased reactivity of PMSF over BSF for the mouse AChE and the close similarity in structure of the two enzymes, wild-type *T. californica* AChE is inactivated by BSF, whereas PMSF has no effect on this enzyme. The object of this study was to assess the influence of various residues on this difference in reactivity.

The k_2K_I values confirm the fact that wild-type *T. californica* AChE is not inactivated by PMSF at all. Mouse AChE, on the other hand, is inactivated by PMSF, with a k_2/K_I value of 960 M^{-1} min^{-1}. The k_2/K_I values for mouse Y330A and Y330F are of the same magnitude as the wild-type mouse AChE; this indicates that mutating the mouse enzyme at position 330 does not significantly affect its PMSF sensitivity. This is in contrast to the large change in specificity found for huperzine binding to the enzyme on making these same mutations (Saxena et al., 1994). PMSF must bind to the enzyme at a location different than that of huperzine.

PMSF inactivates mouse BChE with a k_2/K_I value approximately 8- to 9-fold greater than that of mouse AChE (Table 3), indicating that the large acyl pocket of BChE is important for accommodating PMSF. We therefore investigated the effects on PMSF inactivation of mutating positions 288 and 290 in both mouse and *T. californica* AChEs. The mutations F288L and F290I convert mouse AChE residues to those normally present in mouse BChE. If these positions are those primarily responsible for the difference in inactivation between AChE and BChE, then we would expect that mutating mouse AChE to residues present in BChE at these positions would increase inactivation rates. Indeed, the k_2/K_I values of F288L and F290I, which are 16,000 and 56,000 M^{-1} min^{-1} in the presence of substrate, respectively, show this expected increase in rate of inactivation (Table 3). Unexpectedly, however, *T. californica* F288L/F290V showed a much more dramatic increase in inactivation by PMSF. In fact, these mutations caused the enzyme to change its behavior from completely insensitive to PMSF to a k_2/K_I value of 6000 M^{-1} min^{-1}, behavior in the same range as the mouse acyl pocket mutants. We expected that the difference in sensitivity of mouse and *T. californica* AChE to PMSF would be a result of differences in amino acid sequence. However, mutation of residues that were the same in both species resulted in loss of the difference in sensitivity.

PMSF Inhibition of BSF Inactivation. It remains unclear why *T. californica* AChE is insensitive to PMSF while it is inactivated by BSF. The difference in inactivation might be

TABLE 3
PMSF inactivation of cholinesterase mutants. Average of at least two independent experiments.

Enzyme	Absence of Substrate			Presence of Substrate		
	K_1 (mM)	k_2 (min^{-1})	k_2/K_1 (M^{-1} min^{-1})	K_1 (mM)	k_2 (min^{-1})	k_2/K_1 (M^{-1} min^{-1})
WT *T. californica*	ND	ND	0 ± 16	ND	ND	0[b]
WT mouse	0.7 ± 0.2	0.6 ± 0.2	1,200 ± 700	0.3 ± 0.1[a]	0.3 ± 0.1[a]	960 ± 40
Y330A mouse	1.6 ± 0.3	1.3 ± 0.2	800 ± 300	ND	ND	1,000 ± 300
Y330F mouse	1.4 ± 0.7	1.2 ± 0.5	900 ± 800	ND	ND	380 ± 80
WT mouse BCHe	0.004 ± 0.009	0.04 ± 0.04	10,000 ± 30,000	ND	ND	9,000 ± 1,000
F288L mouse	0.06 ± 0.07	0.3 ± 0.3	6,000 ± 12,000	0.03 ± 0.01	0.4 ± 0.3	16,000 ± 2,000
F290I mouse	ND	ND	7,500 ± 370	0.0021 ± 0.0002	0.12 ± 0.02	56,000 ± 2,000
F288L/F290V *T. californica*	ND	ND	3,800	0.2 ± 0.1	1.0 ± 0.8	6,000 ± 1,000

[a] Data from one experiment only used to determine this variable.
[b] Lack of inactivation precluded error analysis.
WT, wild-type; ND, not determined.

caused by one of two factors: 1) PMSF may be unable to bind in the *T. californica* active site gorge or 2) PMSF may be unable to orient within the gorge to sulfonylate the *T. californica* AChE. To determine which factor is responsible, *T. californica* AChE was assayed with both PMSF and BSF simultaneously to observe the ability of PMSF to competitively inhibit BSF inactivation. BSF (1.5 mM) and various concentrations of PMSF were added to the enzyme solution at time zero, after which the same procedure (described above) was followed to measure BSF inactivation in the absence of substrate. If PMSF cannot bind at all in the active site gorge, we would expect the k' values for BSF to remain constant regardless of the concentration of PMSF. This is exactly what happens (data not shown), indicating that no competitive inhibition is occurring.

The results of the PMSF/BSF competition studies seem to indicate that PMSF fails to inactivate *T. californica* AChE because of an inability to bind in the active site gorge. This is a surprising result, because there are many hydrophobic inhibitors of AChE that are larger than PMSF that are able to enter and bind in the active site gorge. Nevertheless, if PMSF is unable to bind in the gorge, then one of the residues lining the gorge that differ between *T. californica* and mouse AChE should be responsible for blocking the active site. However, altering residues 288 and 290, which lie within the gorge but are the same for mouse and *T. californica*, results in an enzyme that is now able to accommodate PMSF productively within the gorge. This observation suggests that there may be "breathing" motions that are different between mouse and *T. californica* that restrict PMSF orientation in the native *T. californica* enzyme. These breathing motions may be less restricted in the F288L/F290V *T. californica* mutant, thus permitting accomodation of PMSF. The source of the differences in these motions between native mouse and *T. californica* enzymes may be difficult to find, because they need not be the result of differing amino acid residues in the active site gorge itself, but in more distant parts of the protein. Although we previously thought that a complete study of the active-site residues that are different in the mouse and *T. californica* enzymes would clarify the structural basis for the difference in PMSF specificity, we now think that looking at mutations that change the breathing characteristics of the enzyme may be more productive. Morel et al. (1999) have reported that a mutation in the *T. californica* enzyme, L282A (a residue that is the same in Torpedo and mouse), has decreased temperature stability (decreased enthalpy of activation for heat denaturation) that also confers increased reactivity of C231 with thiol reagents. This seems to suggest an increase in breathing of the mutant so as to allow thiol reagents access to C231 (a buried residue). We have preliminary data showing that *T. californica* L282A is inactivated by PMSF (manuscript in preparation). This supports the idea that the thermal instability reflects increased breathing motions and that these motions are connected with PMSF sensitivity.

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References

Axelsen P, Harel M, Silman I and Sussman J (1994) Structure and dynamics of the active site gorge of acetylcholinesterase: Synergistic use of molecular dynamics simulation and X-ray crystallography. *Protein Sci* **3:**188–197.
Barnett P and Rosenberry T (1978) Inactivation of *Electrophorus electricus* acetylcholinesterase by benzenemethane sulfonyl fluoride. *Arch Biochem Biophys* **190:** 202–205.
Bourne Y, Taylor P, Bougis PE and Marchot P (1999) Crystal structure of mouse acetylcholinesterase: A peripheral site-occluding loop in a tetrameric assembly. *J Biol Chem.* **274:**2963–2970.
Ellman G, Courtney KD, Andres JV and Featherstone R (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7:**88–95.
Fahrney D and Gold A (1963) Sulfonyl fluorides as inhibitors of esterases. I. Rates of reaction with acetylcholinesterase, α-chymotrypsin, and trypsin. *J Am Chem Soc* **85:**997–1000.
Harel M, Sussman JL, Krejci E, Bon S, Chanal P, Massoulie J and Silman I (1992) Conversion of acetylcholinesterase to butyrylcholinesterase: Modeling and mutagenesis. *Proc Natl Acad Sci USA* **89:**10827–10831.
Hart GJ and O'Brien RD (1973) Recording spectrophotometric method for determination of dissociation and phosphorylation constants for the inhibition of acetylcholinesterase by organophosphates in the presence of substrate. *Biochemistry* **12:**2940–2945.
Millard CB and Broomfield CA (1995) Anticholinesterases: Medical applications of neurochemical principles. *J Neurochem* **64:**1909–1918.
Morel N, Bon S, Greenblatt HM, Belle DV, Wodak SJ, Sussman JL, Massoulié J and Silman I (1999) Effect of mutations within the peripheral anionic site on the stability of acetylcholinesterase. *Mol Pharmacol* **55:**982–992.
Moss D and Fahrney D (1978) Kinetic analyses of differences in brain acetylcholinesterase from fish or mammalian sources. *Biochem Pharmacol* **27:**2693–2698.
Radic Z, Pickering NA, Vellom DC, Camp S and Taylor P (1993) Three distinct domains in the cholinesterase molecule confer selectivity for acetyl- and butyrylcholinesterase inhibitors. *Biochemistry* **32:**12074–12084.
Saxena A, Qian N, Kovach I, Kozikowski AP, Pang YP, Vellom D, Radic Z, Quinn D, Taylor P and Doctor B (1994) Identification of amino acid residues involved in the binding of huperzine A to cholinesterases. *Protein Sci* **3:**1770–1778.
Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L and Silman I (1991) Atomic structure of acetylcholinesterase from *Torpedo californica*: A prototypic acetylcholine-binding protein. *Science (Washington DC)* **253:**872–878.
Taylor P (1998) Development of acetylcholinesterase inhibitors in the therapy of Alzheimer's disease. *Neurology* **51**(Suppl 1)**:**S30–S35.
Vellom D, Radic Z, Li Y, Pickering N, Camp S and Taylor P (1993) Amino acid residues controlling acetylcholinesterase and butyrylcholinesterase specificity. *Biochemistry* **32:**12–17.
Voet D and Voet JG (1995) *Biochemistry.* 2nd ed. John Wiley and Sons, New York.

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