VOLUME 122, NUMBER 13
APRIL 5, 2000

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Delineation and Decomposition of Energies Involved in Quaternary Ammonium Binding in the Active Site of Acetylcholinesterase

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Received September 15, 1999

Abstract: The quaternary ammonium binding locus in the active site of mammalian acetylcholinesterase is subtended by the side chains of Trp86, Tyr133, Glu202, and Tyr337. Linear free-energy relationships define the interactions involved in molecular recognition by mouse acetylcholinesterase of the quaternary ammonium moiety of ligands. For substrates $CH_3C(=O)XCH_2CH_2Y$ [X = O, Y = $CHMe_2$, or CH_2CH_3; X = S, Y = H, NH^+Me_2, or N^+Me_3] and trifluoroacetophenone transition state analogue inhibitors *m*-$YC_6H_4C(=O)CF_3$ [Y = H, Me, Et, *i*Pr, *t*Bu, CF_3, NH_2, NO_2, NMe_2, or N^+Me_3], $\log(k_{cat}/K_m)$ and pK_i depend linearly on the molar refractivity, but not the hydrophobicity, of the substituents Y. These correlations indicate that, in the acylation stage of catalysis, interactions in the quaternary ammonium binding locus stabilize the tetrahedral intermediate (as modeled by transition state analogue affinity) by (5×10^5)-fold ($\Delta\Delta G^{TI} = -32.5$ kJ mol^{-1}) and the transition state by (2×10^4)-fold ($\Delta\Delta G^{‡} = -24.5$ kJ mol^{-1}). To evaluate the contribution of cation-π interactions, Trp86 was converted into Tyr, Phe, and Ala by site-specific mutagenesis. For this set of enzymes, a linear free-energy relationship is observed between the pK_i values for inhibitions by the respective neutral and cationic transition state analogue inhibitors, *m*-*tert*-butyltrifluoroacetophenone and *m*-(*N*,*N*,*N*-trimethylammonio)-trifluoroacetophenone, which indicates that the free energy released on interaction of the quaternary ammonium moiety with Trp86 arises about equally from cation-π and charge-independent interactions.

Introduction

A fundamental challenge in the biophysical attribution of the structural origins of enzyme catalytic power is to describe the nature and free energetic consequences of molecular interactions that stabilize the transition state, and that thereby effect rate acceleration. Acetylcholinesterase (AChE[1]) is an attractive target for such endeavors, since the catalytic power of the enzyme is highly developed. AChE catalysis is minimally described by the following three-step mechanism, in which E, EA, and F are the free enzyme, Michaelis complex, and acylenzyme intermediate, respectively, A is the substrate, and P and Q are the respective choline and acetate products:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} F + P \xrightarrow[k_5]{H_2O} E + Q$$

$$k_{cat}/K_m = \frac{k_1 k_3}{k_2 + k_3} \quad (1)$$

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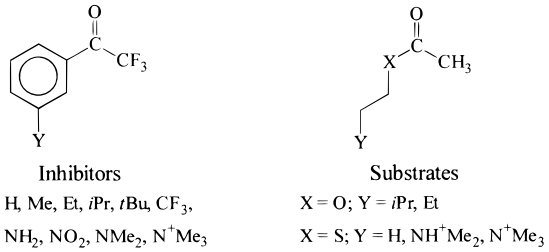
10.1021/ja9933588 CCC: $19.00
Published on Web 03/16/2000

Scheme 1. Substrates and Inhibitors of Mouse Acetylcholinesterase



Equation 1 shows that the bimolecular rate constant k_{cat}/K_m takes one from the free E and free A reactant state through the transition state of the first irreversible step (i.e. k_3) of the mechanism. Hence, k_{cat}/K_m can be viewed as an acylation rate constant. Values of the acylation rate constant k_{cat}/K_m that are $>10^9$ M^{-1} s^{-1} have been reported for (acetylthio)choline (ATCh) turnover.[2] Moreover, AChE accelerates acetylcholine turnover by at least 10^{13}-fold,[3] which corresponds to -74 kJ mol^{-1} of free energy of stabilization of the transition state.[4] The purpose of this report is to describe a multifarious array of structure−activity relationships that characterize the nature of the physical forces that mouse AChE utilizes in molecular recognition of the quaternary ammonium moiety of substrates and trifluoroacetophenone inhibitors. The crux of this characterization is the determination of free energies of stabilization that provide a substantial fraction of the catalytic power of AChE. Moreover, unique structure−activity relationships are described in which the enzyme structure is varied, and which initiate efforts to attribute components of the above-mentioned free energies of stabilization to individual active site amino acid constituents of the quaternary ammonium binding locus.

Results and Discussion

Linear Free-Energy Relationships for Catalysis by and Inhibition of Wild-Type AChE. Scheme 1 shows the families of substituted trifluoroacetophenone inhibitors and acetate ester substrates that were used to gauge molecular recognition in the quaternary ammonium binding locus of mouse AChE. Table 1 lists enzyme kinetic parameters for the substrates and inhibition constants for the inhibitors for wild-type mouse AChE-catalyzed reactions. Previous work indicated that the free energies of interaction in the quaternary ammonium binding loci of *T. californica* and *E. electrophorus* AChEs depend linearly on the molar refractivity of the substituent, which was interpreted as indicating that dispersion interactions contribute to molecular recognition of the quaternary ammonium function.[5] However, a powerful case has been developed in recent years, particularly by Dougherty and co-workers,[6] that aromatic functions interact with organic cations via stabilizing cation-π interactions.[7] We describe below mutagenesis experiments that indicate that both cation-π and dispersion interactions contribute to molecular recognition in the quaternary ammonium binding locus of mouse AChE.

Table 1. Acylation Rate Constants and Inhibition Constants for Wild-Type Mouse Acetylcholinesterase

substrates			inhibitors[a]	
X	Y	k_{cat}/K_m (M^{-1} s^{-1})	Y	K_i
O	*i*Pr	$1.2 \pm 0.2 \times 10^5$	H	46 ± 2 nM
O	Et	8700 ± 400	Me	2.85 ± 0.05 nM
S	H	2200 ± 300	Et	0.51 ± 0.03 nM
S	NH^+Me_2	$3.1 \pm 0.5 \times 10^6$	*i*Pr	15.6 ± 0.7 pM
S	N^+Me_3[c]	$6 \pm 3 \times 10^7$	*t*Bu	3.7 ± 0.6 pM[b]
S	N^+Me_3[c]	$4 \pm 1 \times 10^7$	CF_3	0.78 ± 0.03 nM
			NH_2	69.8 ± 0.6 nM
			NO_2	3.30 ± 0.07 nM
			NMe_2	14.4 ± 0.8 pM
			N^+Me_3	3.7 ± 0.4 fM[c]
			N^+Me_3	4.7 ± 0.4 fM[c]

[a] K_i values for trifluoroketone inhibitors refer to the free ketone, and consequently have been corrected for equilibrium hydration, as described by Nair et al.[5] [b] Taken from Radić et al.[26c] [c] Replicate values were determined for the substrate and inhibitor that have $Y = N^+Me_3$.

As Figure 1A shows, linear free-energy correlations indicate that both catalysis and inhibition depend on the molar refractivity of the substituents Y. The plot for inhibitors was constructed by first fitting data to the following multiple linear free-energy relationship:

$$pK_i = \rho\sigma_m + m\cdot MR + C \qquad (2)$$

The constants ρ and m are the sensitivities of inhibitor potency pK_i to the electronic substituent constant (σ_m) and molar refractivity (MR) of the meta substituent. The value of ρ, 1.6 $\pm$ 0.5, is similar to that determined from a correlation of $\log K_{Hyd}$ versus σ_m (not shown), where K_{Hyd} is the equilibrium constant for hydration of the various trifluoroacetophenone inhibitors. This similarity is consistent with the observation that, in the X-ray structure of the complex of *T. californica* AChE and the inhibitor *m*-(*N*,*N*,*N*-trimethylammonio)trifluoroacetophenone (TMTFA), the γ-O of Ser200 of the active site is within covalent bond distance of the carbonyl carbon of the inhibitor.[8] The linear correlation for inhibition in Figure 1A is plotted as $pK_i - \rho\sigma_m$ versus molar refractivity, a presentation that emphasizes the dependence of inhibitor potency on interaction in the quaternary ammonium binding locus only, and which facilitates comparison with the correlation of $\log(k_{cat}/K_m)$ and molar refractivity for substituted substrates. The linear free-energy correlations of Figure 1A suggest that mouse AChE, like the marine AChEs,[5] also utilizes dispersion interactions to stabilize ligand complexes.

(1) Abbreviations used: AChE, acetylcholinesterase; Ala, alanine; ATCh, (acetylthio)choline; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Glu, glutamic acid; MEPQ, 7-(methylethoxyphosphinyloxy)-*N*-methyl quinolinium iodide; MR, molar refractivity; Phe, phenylalanine; Ser, serine; TBTFA, *m*-*tert*-butyltrifluoroacetophenone; TMTFA, *m*-(*N*,*N*,*N*-trimethylammonio)trifluoroacetophenone; Trp, tryptophan; Tyr, tyrosine.

(2) Nolte, H.-J.; Rosenberry, T. L.; Neumann, E. *Biochemistry* **1980**, *19*, 3705−3711.

(3) Quinn, D. M. *Chem. Rev.* **1987**, *87*, 955−979.

(4) The free energy of stabilization of the transition state was calculated as $\Delta\Delta G^{\ddagger} = -RT \ln C$, where $R = 8.314$ J K^{-1} mol^{-1}, $T = 298$ K, and C is the catalytic acceleration. All free energy calculations in this paper utilize $T = 298$ K and 1 M standard states for all relevant species.

(5) Nair, H. K.; Seravalli, J.; Arbuckle, T.; Quinn, D. M. *Biochemistry* **1994**, *33*, 8566−8576.

(6) (a) Dougherty, D. A.; Stauffer, D. A. *Science* **1990**, *250*, 1558−1560. (b) Kearney, P. C.; Mizoue, R. S.; Kumpf, R. A.; Forman, J. E.; McCurdy, A.; Dougherty, D. A. *J. Am. Chem. Soc.* **1993**, *115*, 9907−919. (c) Mecozzi, S.; West, A. P.; Dougherty, D. A. *J. Am. Chem. Soc.* **1996**, *118*, 2307−2308. (d) Dougherty, D. A. *Science* **1996**, *271*, 163−168. (e) Ma, J. C.; Dougherty, D. A. *Chem. Rev.* **1997**, *97*, 1303−1324.

(7) Shafferman and co-workers have championed the view that cation-π interactions with Trp86 are important for molecular recognition by AChE of quaternary ammonium ligands: (a) Ordentlich, A.; Barak, D.; Kronman, C.; Flashner, Y.; Leitner, M.; Segall, Y.; Ariel, N.; Cohen, S.; Velan, B.; Shafferman, A. *J. Biol. Chem.* **1993**, *268*, 17083−17095. (b) Ordentlich, A.; Barak, D.; Kronman, C.; Ariel, N.; Segall, Y.; Velan, B.; Shafferman, A. *J. Biol. Chem.* **1995**, *270*, 2083−2091.

(8) Harel, M.; Quinn, D. M.; Nair, H. K.; Silman, I.; Sussman, J. L. *J. Am. Chem. Soc.* **1996**, *118*, 2340−2346.

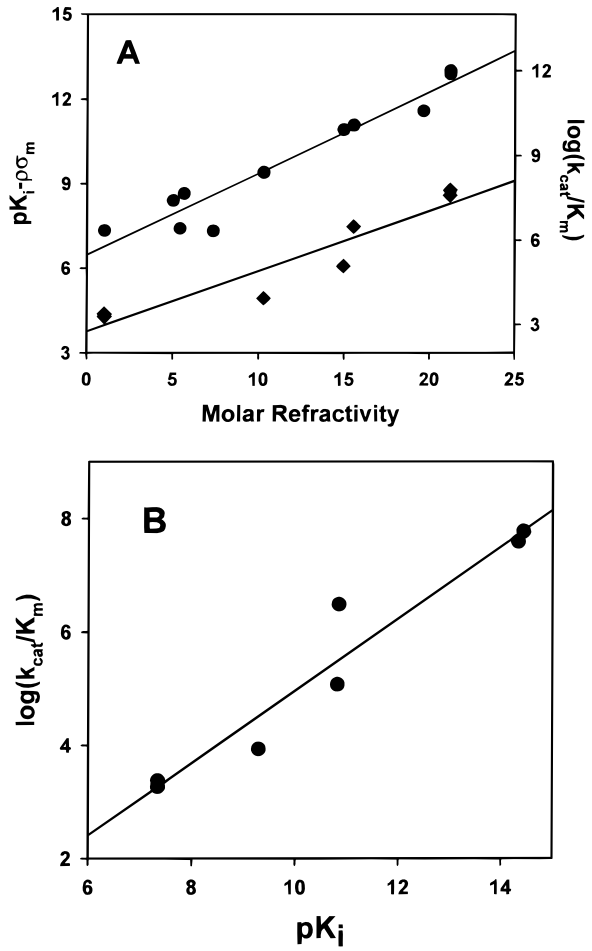


Figure 1. Linear free-energy correlations for substrates and inhibitors of mouse AChE. (A) Circles and diamonds are for inhibition by meta-substituted trifluoroacetophenones and for substrate hydrolysis, respectively. Inhibition data were fit to eq 2 of the text to give $m = 0.29 \pm 0.03$, $\rho = 1.6 \pm 0.5$, $C = 6.5 \pm 0.4$, and $r^2 = 0.945$. A rearranged form of eq 2 that emphasizes the linear free-energy relationship between inhibitor potency and molar refractivity of inhibitor substituents is plotted, as described in the text. Substrate hydrolysis data were fit to the following equation: $\log(k_{cat}/K_m) = m\cdot\mathrm{MR} + C$. The parameters of the fit are $m = 0.21 \pm 0.03$, $C = 2.8 \pm 0.5$, and $r^2 = 0.886$. (B) Correlation between catalytic rate constant and inhibitor potency. The solid line is a fit to the following equation: $\log(k_{cat}/K_m) = m\cdot\mathrm{p}K_i + C$. The parameters of the fit are $m = 0.64 \pm 0.08$, $C = -1.4 \pm 0.8$, and $r^2 = 0.934$.

On the other hand, correlations with the hydrophobic substituent constant are linear for alkyl substituents but fail for nitrogen-containing substituents (plot not shown), as observed previously for marine AChEs.[5] However, one cannot exclude the hydrophobic effect altogether from contributing to molecular recognition in the quaternary ammonium binding locus, since a multiple linear free-energy relationship among pK_i, σ_m, and the substituent surface area performs nearly as well as the fit to eq 2 described above.[9] However, the sensitivity of pK_i to substituent surface area is -270 ± 30 J mol^{-1} Å^{-2}, a value that is well above estimates for the transfer of hydrophobic molecular fragments from aqueous solution to neat organic phases.[10] This analysis suggests that the dependence of ligand affinity on molar refractivity (or surface area) for interaction in the quaternary ammonium binding locus of AChE is a result of the interplay of various factors, which include the classical entropy-driven hydrophobic effect as well as short-range van der Waals (dispersion) interactions. The factor contributed by the charge of the ligand is delineated later.

Figure 1B shows that, as expected, there is a linear relationship between $\log(k_{cat}/K_m)$ and pK_i. The slope of this Thompson−Bartlett plot,[11] $m = 0.64 \pm 0.08$, indicates that amino acids of the mouse AChE active site that stabilize the tetrahedral trifluoroketone complex also stabilize the acylation transition state, albeit to a lesser extent. Moreover, these results suggest that the tetrahedral intermediate in the acylation stage of catalysis, to which the trifluoroketone complex bears closest structural resemblance, is stabilized by the quaternary ammonium binding locus more than is the acylation transition state. The overall change in k_{cat}/K_m as one proceeds from Y = H to Me_3N^+ is (2×10^4)-fold, while the overall change in K_i across the series of inhibitors is (1.1×10^7)-fold. For inhibitors, the multiple linear free energy correlation of Figure 1A indicates that, of the (1.1×10^7)-fold potency range, a factor of 5×10^5 arises from interactions in the quaternary ammonium binding locus. The corresponding substituent effects on the stabilities of the acylation transition state and transition state analogue complex are $\Delta\Delta G^{\ddagger} = -24.5$ kJ mol^{-1} and $\Delta\Delta G^{TI} = -40.2$ kJ mol^{-1}, respectively; interactions in the quaternary ammonium binding locus contribute a factor of -32.5 kJ mol^{-1} to $\Delta\Delta G^{TI}$.

Linear Free-Energy Relationships for Wild-Type and Mutant Acetylcholinesterases. The quaternary ammonium binding locus whose function is gauged by the above-described experiments consists of the amino acid side chains of Trp86, Tyr133, Glu202, and Tyr337[8,12] and three water molecules, as shown in Figure 2.[8] The closest interactions between AChE and the quaternary ammonium function involve Trp86 and Glu202. Free energy relationships for native mouse AChE, here denoted Trp86, and the mutants Trp86Tyr, Trp86Phe, and Trp86Ala provide a method for delineating the contributions of W86 of this binding locus to the transition state analogue binding free energies alluded to in the preceding paragraph. Michaelis−Menten kinetic parameters and transition state analogue inhibition constants are given in Table 2 for the three Trp86 mutants of mouse AChE. Mouse Trp86Ala AChE has a k_{cat}/K_m that is 1500-fold less than that of the native enzyme. Therefore, interaction with Trp86 contributes 18 kJ mol^{-1} to stabilization of the acylation transition state. This free energy change is about 75% of that determined above for native mouse AChE-catalyzed hydrolysis of substituted substrates, which provides an indication of the dominant contribution of Trp86 to molecular recognition in the quaternary ammonium binding locus.

(9) Inhibition data were fit to the following equation, in which A is the surface area of the *meta* substituent: $\mathrm{p}K_i = \rho\sigma_m + a\cdot A + C$. The parameter values determined by the fit were $\rho = 2.2 \pm 0.6$, $a = 0.048 \pm 0.006$ Å^{-2}, and $C = 4.5 \pm 0.7$. The correlation coefficient for this fit was $r = 0.962$, only marginally less than $r = 0.965$ for the fit to eq 2. The free energy per substituent surface area for transfer from aqueous solution to the active site of AChE is $-2.3025RT\cdot a = -270 \pm 30$ J mol^{-1} Å^{-2}.

(10) (a) Reynolds et al. estimated that the free energy change per solute surface area for transfer of hydrocarbons from aqueous medium to a hydrocarbon solvent is in the range -20 to -25 cal mol^{-1} Å^{-2} (-84 to -105 J mol^{-1} Å^{-2}): Reynolds, J. A.; Gilbert, D. B.; Tanford, C. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 2925−2927. (b) Herman reported a value of -30 cal mol^{-1} Å^{-2} (-126 J mol^{-1} Å^{-2}) for transfer of alkylbenzenes from aqueous medium to neat aromatic hydrocarbon phases, determined from solubility measurements: Herman, R. B. *J. Phys. Chem.* **1972**, *76*, 2754−2759. (c) A value for the hydrophobic effect of -47 cal mol^{-1} Å^{-2} (-197 J mol^{-1} Å^{-2}) arises when solute−solvent size differences are taken into account: Sharp, K. A.; Nicholls, A.; Fine, R. F.; Honig, B. *Science* **1991**, *252*, 106−109.

(11) (a) Thompson, R. C. *Biochemistry* **1973**, *12*, 47−51. (b) Bartlett, P. A.; Marlowe, C. *Biochemistry* **1983**, *22*, 4618−4624.

(12) Quinn, D. M. In *Comprehensive Toxicology*; Vol. 3, Biotransformation; Guengerich, F. P., Ed.; Elsevier: New York, 1997; pp 243−264.

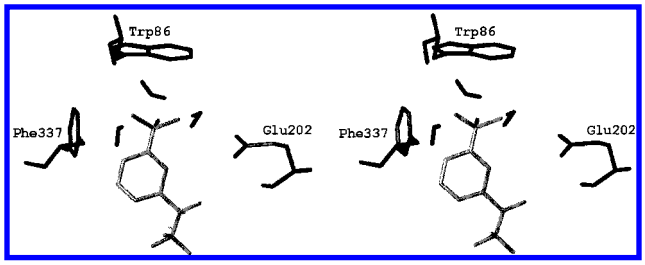


Figure 2. Crossed stereoview of the interaction of TMTFA in the quaternary ammonium binding locus of *Torpedo californica* AChE.[8] The residues that make close contacts with the quaternary ammonium function of TMTFA include Trp86(*84*), Phe337(*330*), Glu202(*199*), and three water molecules. The sequence numbers that follow the amino acid abbreviation are those of mammalian and *Torpedo californica* AChEs, respectively, the latter in italics and parentheses.[29] The mammalian AChE numbering scheme is used in the figure. In mammalian AChE, Tyr replaces Phe at position 337.

Table 2. Acylation Rate Constants and Inhibition Constants For Mutant Mouse Acetylcholinesterases

enzyme	k_{cat}/K_m (M^{-1} s^{-1})[a]	K_i($Y = t$Bu)[b]	K_i($Y = N^+Me_3$)[b]
W86[c]	$6 \pm 3 \times 10^7$	3.7 ± 0.6 pM	3.7 ± 0.4 fM
	$4 \pm 1 \times 10^7$		4.7 ± 0.4 fM
W86F	$3.4 \pm 0.4 \times 10^6$	6 ± 1 pM	0.11 ± 0.01 pM
W86Y	$2.0 \pm 0.2 \times 10^6$	33 ± 8 pM	0.37 ± 0.04 pM
W86A	$3.2 \pm 0.8 \times 10^4$	80 ± 30 pM	6.7 ± 0.3 pM

[a] The bimolecular rate constant k_{cat}/K_m is that for turnover of (acetylthio)choline, determined under the experimental conditions outlined in the Methods subsection of the Experimental Section. [b] Data for the uncharged inhibitor, *m-tert*-butyltrifluoroacetophenone, are taken from Radić et al.[26c] K_i values for trifluoroketone inhibitors refer to the free ketone, and consequently have been corrected for equilibrium hydration, as described by Nair et al.[5] [c] Data for wild-type (W86) AChE are reproduced from Table 1.

The slope of the linear free energy relationship in Figure 3A between $\log(k_{cat}/K_m)$ for turnover of ATCh and pK_i for inhibition by TMTFA is $m = 1.0 \pm 0.1$. Hence, the sensitivities of catalysis and transition state analogue binding to variation of the AChE substituent at position 86 are the same. This comparison indicates that, inasmuch as the tetrahedral trifluoroketone complex resembles the tetrahedral intermediate in the acylation stage of catalysis,[8] the influence of Trp86 on the stabilities of the tetrahedral intermediate and the acylation transition state is the same. Figure 3B shows that, as for variation of substrate and inhibitor substituents in Figure 1A, there is a linear free-energy relationship between $\log(k_{cat}/K_m)$ and molar refractivity of the varied amino acid at position 86. This correlation contains data not only from the current study but also from k_{cat}/K_m values reported by Shafferman et al.[13] for wild-type human AChE and the corresponding Trp86Phe, Trp86Ala, and Trp86Glu mutants. Inclusion of the human AChE data underscores the general applicability of the linear free-energy correlation to probing molecular recognition in the quaternary ammonium binding locus.

Figure 4 demonstrates that more than hydrophobic and dispersion interactions are involved in molecular recognition in the quaternary ammonium binding locus. For wild-type AChE and the three Trp86 mutants, a linear free-energy relationship is observed between pK_i values for trifluoroacetophenone transition state analogue inhibitors that have isosteric uncharged (*m-tert*-butyl) and charged (*m*-trimethylammonio) substituents.

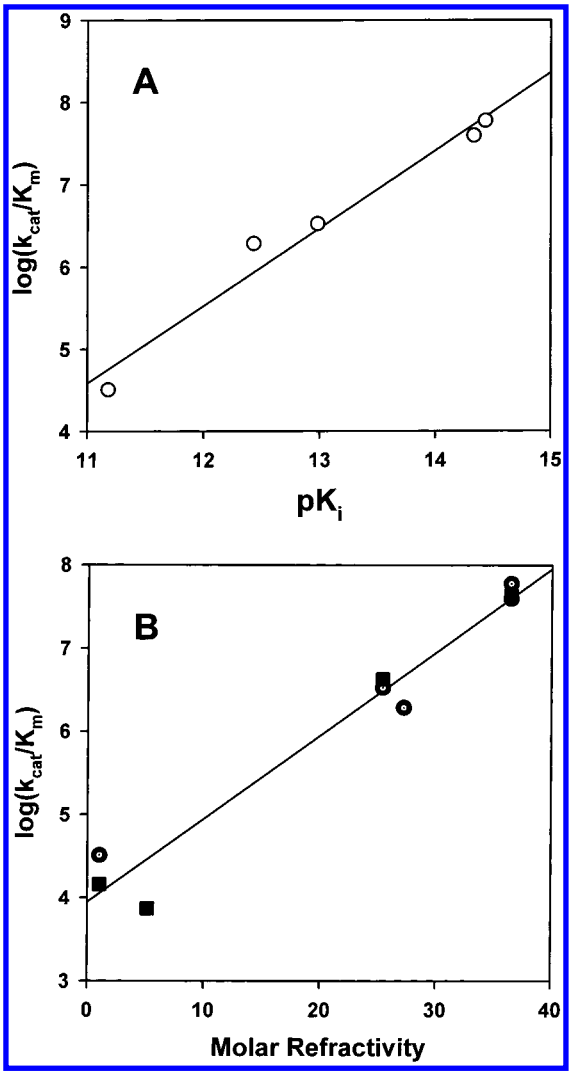


Figure 3. Linear free-energy correlations for wild-type and mutant mouse AChEs. Except where noted, the data used to construct these correlations are contained in Tables 1 and 2. (A) The bimolecular rate constant for AChE-catalyzed hydrolysis of ATCh is correlated with inhibitory potency of TMTFA. The points from left to right are those for Trp86Ala, Trp86Tyr, Trp86Phe, and wild-type AChE (rightmost two points). The solid line is a fit to the equation in the legend of Figure 1B, and the parameters of the fit are $m = 1.0 \pm 0.1$, $C = -6 \pm 1$, and $r^2 = 0.969$. (B) The bimolecular rate constant for AChE-catalyzed hydrolysis of ATCh is correlated with the molar refractivity of the amino acid side chain at position 86. Circles are from this study, and from left to right are for Trp86Ala, Trp86Tyr, Trp86Phe, and wild-type mouse AChEs. Squares were calculated from data of Shafferman et al.[13] for native and mutant human AChEs, and from left to right are for Trp86Ala, Trp86Glu, Trp86Phe, and wild-type enzymes. The solid line is a fit to the equation in the legend of Figure 1A, and the parameters are $m = 0.100 \pm 0.008$, $C = 3.9 \pm 0.2$, and $r^2 = 0.960$.

The slope of this correlation is $m = 0.42 \pm 0.07$. Since the uncharged and charged substituents have similar molar refractivities (19.62 and 21.20, respectively[14]), a slope of 0.93 is expected if dispersion interactions alone are important for quaternary ammonium recognition by Trp86. That $m = 0.42$ suggests that the free energy released on interaction of the quaternary ammonium moiety with Trp86 is about evenly divided between contributions that are dependent on substituent charge on one hand and those that are independent of charge

(13) Shafferman, A.; Ordentlich, A.; Barak, D.; Kronman, N.; Ariel, M.; Leitner, M.; Segall, Y.; Bromberg, A.; Reuveny, S.; Marcus, D.; Bino, T.; Lazar, A.; Cohen, S.; Velan, B. In *Enzymes of the Cholinesterase Family*; Quinn, D. M., Balasubramanian, A. S., Doctor, B. P., Taylor, P., Eds.; Plenum: New York and London, 1995; pp 189−196.

(14) Hansch, C.; Leo, A. *Substituent Constants for Correlation Analysis in Chemistry and Biology*; John Wiley: New York, 1979.

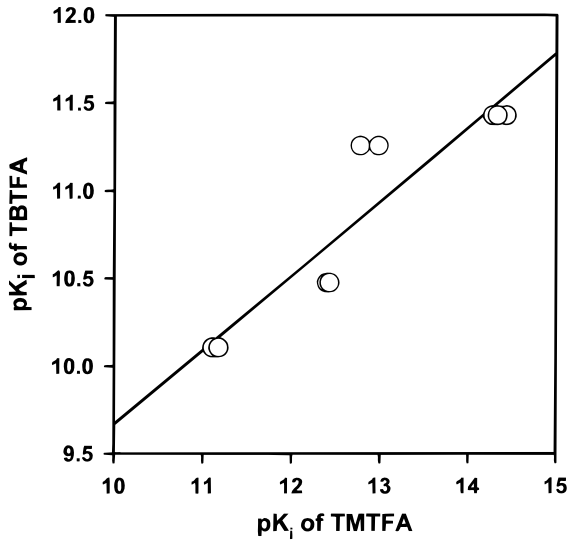


Figure 4. Linear free-energy correlation between inhibitory potencies of uncharged and cationic transition state analogue inhibitors of wild-type and mutant mouse AChEs. pK_i values for the *m-tert*-butyl and *m*-trimethylammonio inhibitors (TBTFA and TMTFA, respectively) were calculated from data in Tables 1 and 2 and in Radić et al.[26c] The clusters of points are, from left to right, for Trp86Ala, Trp86Tyr, Trp86Phe, and wild-type enzymes. The solid line is a fit to the following equation: pK_i(TBTFA) = m·pK_i(TMTFA) + C. The parameters of the fit are $m = 0.42 \pm 0.07$, $C = 5.4 \pm 0.9$, and $r^2 = 0.854$.

but dependent on the size of the substituent on the other. It is reasonable to conclude that the 18 kJ mol^{-1} change in free energy on interaction with Trp86 is about evenly split between cation-π and dispersion/hydrophobic interactions.

Comparison with Other Studies. The contribution of charge-dependent interactions to the total interaction free energy for the quaternary ammonium binding site can be estimated in the following way. As mentioned earlier, the affinity range for meta-substituted trifluoroacetophenones is 1.1×10^7, of which a factor of 5.5×10^5 (corresponding $\Delta\Delta G^{TI} = -32.5$ kJ mol^{-1}) arises from interaction with the quaternary ammonium binding locus of AChE. From the K_i values of Table 1 one calculates that the quaternary ammonium inhibitor TMTFA (Y = N^+Me_3) binds more tightly than the isosteric neutral inhibitor TBTFA (Y = *tert*-butyl) by a factor of 880, which corresponds to an interaction free energy difference of −16.8 kJ mol^{-1}. A portion of this higher affinity is due to the Hammett substituent effect on the stability of the hemiketal adduct between the inhibitor and the γ-O of Ser203(*200*). This factor is estimated as $\Delta\Delta G_{Hammett} = -2.303RT\cdot\rho(\sigma_{TMTFA} - \sigma_{TBTFA}) = -10$ kJ mol^{-1}, where σ_{TMTFA} and σ_{TBTFA} are the σ_m values[14] for the *m*-Me_3N^+ and the *m-tert*-butyl substituents of TMTFA and TBTFA, respectively. This calculation leaves a difference of −6.8 kJ mol^{-1} of interaction free energy that arises specifically from the positive charge of the quaternary ammonium moiety.

The affinity of wild-type mouse AChE for the quaternary ammonium inhibitor TMTFA is 1600-fold greater than that of the Trp86Ala mutant, which corresponds to a change in interaction free energy of −18.3 kJ mol^{-1}. The linear free energy correlation of Figure 4 suggests that −10.6 kJ mol^{-1} of this free energy can be attributed to the charge of the quaternary ammonium inhibitor. Therefore, this estimate and that in the preceding paragraph suggest that positive charge accounts for ∼ −7 to −11 kJ mol^{-1} of the energy that is released on binding of ligands to the quaternary ammonium binding locus of AChE.

It is instructive to compare the interaction energies estimated in the preceding two paragraphs with similar estimates from other investigations. Pressman et al. studied reactions of antibodies homologous to quaternary ammonium haptenic groups. They found that a hapten that contained the *p*-azophenyltrimethylammonium moiety had a 15.5-fold higher affinity than did the isosteric hapten that contained the *p*-azo-*tert*-butylbenzene moiety. The corresponding Coulomb interaction energy is −6.3 kJ mol^{-1}, a value that is in reasonable agreement with the estimates generated in this paper.

Ab initio calculations of cation-π interactions between Na^+ and aromatics[15] indicate that the energy of complexation is about evenly split between charge-dependent and charge-independent contributions, just as observed experimentally herein. This situation also obtains for calculated energies for interaction of benzene with ammonium ions.[16] The free energy difference of −18.3 kJ mol^{-1} reported herein for binding of TMTFA to wild-type AChE versus the Trp86Ala mutant is in good agreement with calculated free energies of complexation of tetramethylammonium ion and benzene in the gas phase, −17 kJ mol^{-1},[16,17] in aqueous solution, −13.8 kJ mol^{-1},[18] and with the corresponding experimental free energy of association in the gas phase, −14.6 kJ mol^{-1}.[19] From the results described herein, both log(k_{cat}/K_m) and pK_i increase in the sequence Tyr < Phe ≪ Trp for the various aromatic residues at position 86. This trend agrees with the trend in calculated binding energies for complexation of Na^+ and Me_4N^+ with the series of aromatics phenol, benzene, and indole.[20,21]

Zhong et al.[22] used the in vivo nonsense-suppression method to introduce unnatural amino acids into specific positions in the nicotinic acetylcholine receptor. Large effects of unnatural amino acid mutagenesis of Trp149 of the α subunit of the receptor were noted, whereas mutagenesis at alternate Trp residues had diminutive effects. Of particular note was the linear free-energy correlation between the EC_{50} values for receptor interaction with acetylcholine and the cation-π binding energies, calculated by ab initio quantum mechanics, for the side chains of Trp149 and various fluorinated analogues thereof. The range of EC_{50} values was about 2 orders of magnitude, which corresponds to a $\Delta\Delta G$ of ∼ −11 kJ mol^{-1}, again in substantial agreement with estimates generated herein of the interaction energy of the quaternary ammonium moiety with Trp86 of AChE.

Barak et al.[23] recently reported an insightful study of the effect of mutation of Trp86 on the rates of aging of the structurally varied human AChE phosphonyl conjugates of Scheme 2. The first-order aging rate constants decrease in the order Trp86 > Phe86 > Ala86; this trend is observed for all degrees of branching at the β-carbon. However, the range of aging rate constants for Trp86 versus Ala86 AChE decreased systemati-

(15) Electrostatics contribute 58% and 59% to the calculated interaction energies of Na^+ with benzene and phenol, respectively. See ref 6c.

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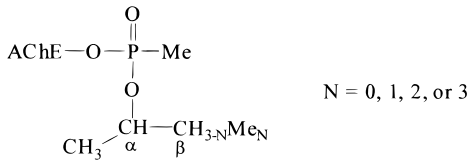
(23) Barak, D.; Ordentlich, A.; Segall, Y.; Velan, B.; Benschop, H. P.; De Jong, L. P. A.; Shafferman, A. *J. Am. Chem. Soc.* **1997**, *119*, 3157−3158. The aging of phosphyl conjugates of AChE is the hydrolytic cleavage of the O−C_α bond of Scheme 2, a reaction that is thought to involve a carbocation intermediate and/or carbocationic transition state.

Scheme 2. Phosphonyl Conjugates of Acetylcholinesterase Studied by Barak et al.[23]



cally with decreasing β branching, from 1120-fold when $N = 3$ to 60-fold when $N = 0$. Barak et al.[23] interpret these results as indicating that aromatic residues at position 86, and in particular Trp, stabilize the carbocationic transition state of the aging reaction by cation-π interactions. For all but the least branched conjugate, $\log(k_a)$ is a linear function of the molar refractivity of the amino acid at position 86; r^2 values are 0.99, 0.98, and 0.97 for $N = 3$, 2, and 1, respectively. The activation energy differences for the wild-type (i.e. Trp86) and Ala86 enzymes are −17.4, −14.7, and −14.7 kJ mol^{-1}, respectively, in good agreement with our estimate of the energetics of interaction of Trp86 with quaternary ammonium ligands.

The studies reported herein, and those of Zhong et al.[22] and Barak et al.,[23] show that linear free-energy relationships (LFERs) are observed either when ligand substituent or enzyme/receptor structure is systematically varied. The use of site-directed mutants to construct LFERs adds an important new tool to the arsenal of approaches for structure−function investigations[24] of cholinesterases.

Experimental Section

Materials. Substrates were used as received from the following sources: (acetylthio)choline iodide, Sigma Chemical Co.; butyl acetate and isoamyl acetate, Aldrich Chemical Co.; ethyl thioacetate, MTM Research Chemicals; 2-(*N*,*N*-dimethylamino)ethyl thioacetate, from a previous study.[5] Trifluoroacetophenone was used as received from Aldrich Chemical Co.; meta-substituted trifluoroacetophenone inhibitors were synthesized and structurally characterized as described previously.[5,25] DTNB and *p*-nitrophenol were used as received from Sigma Chemical Co. Buffer components were used as received from the following sources: $NaH_2PO_4 \cdot H_2O$ and Na_2HPO_4, Sigma Chemical Co.; NaCl, Fisher Scientific. Water for buffer preparation was distilled and then deionized by passage through a Barnstead D8922 mixed-bed ion-exchange column (Sybron Corp.). Recombinant mouse AChEs were expressed and characterized as described by Radić et al.[26] 7-(Methylethoxyphosphinyloxy)-*N*-methyl quinolinium iodide (MEPQ) was obtained *gratis* from Yacov Ashani of the Israel Institute for Biological Research, Ness-Ziona.

Methods. Molar refractivities and σ_m values for inhibitor and substrate substituents were taken from Hansch and Leo.[14] Substituent van der Waals surface areas were calculated by using the MOLCAD feature of the molecular modeling program Sybyl 6.1 (Tripos Associates Inc., St. Louis, MO). Reactions were run at 25.0 ± 0.2 °C in 0.05 M sodium phosphate buffers, pH 7.00, that contained sufficient ATCh and/or NaCl to give an ionic strength (*I*) of 0.13. AChE-catalyzed reactions were monitored on HP8452 or Beckman DU40 UV−visible spectrophotometers. Hydrolyses of the thioesters ATCh, ethyl thioacetate, and 2-(*N*,*N*-dimethylamino)ethyl thioacetate were followed at 412 nm by coupling the released thiol product with DTNB, as described by Ellman et al. for ATCh.[27] Hydrolyses of the oxyesters butyl acetate and isoamyl acetate were followed at 400 nm by a coupled assay that utilized *p*-nitrophenol as the indicator, as previously described.[5] Buffer pH was measured on a Corning Model 125 pH meter that was equipped with a glass combination electrode. Enzyme concentrations were determined by active site titration with MEPQ, as described by Ashani et al.[28] Michaelis−Menten kinetic parameters were determined by fitting initial velocity data to an equation that includes substrate inhibition at high substrate concentrations, as described by Radić et al.[26a] Slow, tight-binding inhibitions of wild-type and mutant mouse AChEs by TMTFA and TBTFA were characterized as described by Nair et al.[5]

Acknowledgment. This work was supported by NIH grants NS21334 to D.M.Q. and GM18360 to P.T.

JA9933588

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