Binding of the Neurotoxin Fasciculin 2 to the Acetylcholinesterase Peripheral Site Drastically Reduces the Association and Dissociation Rate Constants for *N*-Methylacridinium Binding to the Active Site†

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ABSTRACT: The acetylcholinesterase (AChE) active site consists of a gorge 2 nm deep that is lined with aromatic residues. A serine residue near the base of the gorge defines an *acylation site* where an acyl enzyme intermediate is formed during the hydrolysis of ester substrates. Residues near the entrance to the gorge comprise a *peripheral site* where inhibitors like propidium and fasciculin 2, a snake neurotoxin, bind and interfere with catalysis. Like certain other cationic ligands that bind specifically to the acylation site, *N*-methylacridinium can still interact with the acylation site in the AChE−fasciculin 2 complex. At 310 K (37 °C), the equilibrium dissociation constant K_L' for *N*-methylacridinium binding to the complex was 4.0 ± 0.7 μM, less than an order of magnitude larger than the K_L = 1.0 ± 0.3 μM for *N*-methylacridinium interaction with human AChE in the absence of fasciculin 2. To assess whether fasciculin 2 can sterically block access of a ligand to the acylation site, thermodynamic and kinetic constants for the interaction of *N*-methylacridinium with AChE in the presence and absence of fasciculin 2 were measured by fluorescence temperature jump relaxation kinetics. During progressive titration of the enzyme with increasing concentrations of *N*-methylacridinium, a prominent relaxation in the 0.1−1 ms range was observed in the absence of fasciculin 2. When excess fasciculin 2 was added, the prominent relaxation shifted to the 0.3−1 s range. Estimates of total AChE concentrations, K_L, or K_L' from analyses of relaxation amplitudes agreed well with those from equilibrium fluorescence, confirming that the relaxations corresponded to the bimolecular reactions of interest. Further analysis of the relaxation times in the absence of fasciculin 2 gave estimates of the *N*-methylacridinium association rate constant $k_{12} = 8 \times 10^8$ M^{-1} s^{-1} and dissociation rate constant k_{21} = 750 s^{-1} at 310 K (37 °C). For the AChE−fasciculin 2 complex, the corresponding constants were $k_{12}' = 1.0 \times 10^5$ M^{-1} s^{-1} and $k_{21}' = 0.4$ s^{-1}. Thus the rate constants decreased by more than 3 orders of magnitude when fasciculin 2 was bound, consistent with a pronounced steric blockade of *N*-methylacridinium ingress to and egress from the acylation site.

The three-dimensional structure of torpedo acetylcholinesterase (AChE; EC 3.1.1.7) (Sussman *et al*, 1991) offers insights into the high catalytic efficiency of this enzyme toward its physiological substrate acetylcholine. An intriguing feature of the AChE structure is the active site (diagrammed in Figure 1), a narrow gorge lined with aromatic residues that is about 20 Å deep and penetrates nearly to the center of the 70 kDa catalytic subunit. At the base of the gorge is the *acylation site*, where acetylcholine is bound as it acylates the S200 residue (torpedo sequence numbering) during substrate turnover and where H440 and E327 residues participate in a catalytic triad similar to those found in other serine proteases and esterases (E−H−S). Crystal structure analyses shows that certain cationic inhibitors bind selectively to this site (Harel *et al*., 1993). For example, edrophonium is bound with its hydroxyl group making hydrogen bonds to both the $N^{\epsilon 2}$ atom of H440 and the O^{γ} atom of S200 and its quaternary ammonium group adjacent to W84. Tacrine is bound to one side of this site, with the positive charge from its tetrahydroaminoacridinium structure interacting with the indole group of W84.

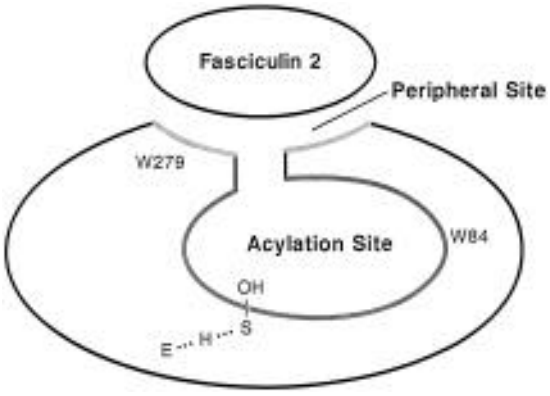

FIGURE 1: Schematic diagram of the sites for ligand binding in AChE.

The *peripheral site* was first defined by cationic inhibitors like propidium that do not compete with edrophonium in binding to the active site (Taylor & Lappi, 1975). Recently the fasciculins, a family of very similar snake venom neurotoxins from mambas (genus *Dendroaspis*), have been shown to interact selectively with this site (see Figure 1).

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These 61-amino acid polypeptides with four disulfide bonds have three-dimensional structures that are very similar to those of the short α-neurotoxins that bind to nicotinic acetylcholine receptors (le Du *et al*., 1992). Studies involving affinity labeling (Weise *et al*., 1990; Schalk *et al*., 1992) and site-specific mutants (Radic *et al*., 1993; Barak *et al*., 1994) indicate that W279 on the rim of the active site gorge is a key component of the peripheral site and is essential for high-affinity fasciculin binding (Radic *et al*., 1994). Residues from at least two other polypeptide loops at the gorge rim also contribute to this site.

While fasciculins and propidium are strictly competitive in binding to the peripheral site (Karlsson *et al*., 1984; Marchot *et al*., 1993; Eastman *et al*., 1995), the affinities of acylation site ligands like edrophonium and *N*-methylacridinium for the complex of AChE and fasciculin 2 are reduced by only about an order of magnitude from their affinities for the free enzyme (Eastman *et al*., 1995). Substrates also can be hydrolyzed by the AChE−fasciculin 2 complex, but the values of the steady state kinetic constants k_{cat} and k_{cat}/K_{app} for the very good substrates acetylthiocholine and phenyl acetate are only 0.2%−2% of those with the free enzyme (Eastman *et al*., 1995; Radic *et al*., 1995). To assess whether fasciculin 2 binding to the peripheral site had a greater effect on substrate access to the acylation site or on steps involving proton transfer once the substrate was bound at the acylation site, deuterium oxide isotope effects on k_{cat}/K_{app} for substrate hydrolysis were measured. These measurements indicated that the toxin had a greater effect on proton transfer step(s) than on substrate dissociation rate constants (Eastman *et al*., 1995).

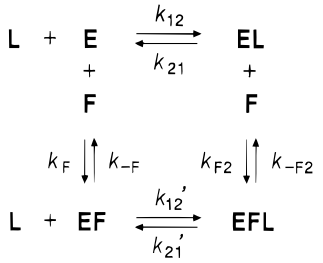
To better understand the consequences of fasciculin binding on active site function, however, it is useful to have a more direct measure of the changes in association and dissociation rate constants for acylation site ligands when fasciculin 2 is bound. The strong fluorescence signal of *N*-methylacridinium is completely quenched when this ligand is bound to the AChE acylation site, and this signal change has been exploited previously to measure association and dissociation rate constants for *N*-methylacridinium and AChE by fluorescence temperature jump relaxation kinetics (Rosenberry & Neumann, 1977; Nolte *et al*., 1980). Applying this technique here, we show that binding of fasciculin 2 to AChE reduces these rate constants by 3−4 orders of magnitude.

EXPERIMENTAL PROCEDURES

Materials. Human erythrocyte AChE was purified as outlined previously and active site concentrations were determined by assuming 410 units/nmol (Rosenberry & Scoggin, 1984; Roberts *et al*., 1987). One unit of AChE activity corresponds to 1 μmol of acetylthiocholine hydrolyzed per min at 23 °C in a standard AChE assay (Ellman *et al*., 1961) as modified by Rosenberry and Scoggin (1984). Purified fasciculin 2 concentrations were determined by molar absorptivity ($\epsilon_{276\ nm} = 4900\ M^{-1}\ cm^{-1}$), and stocks were mixed with bovine serum albumin (to 1 mg/mL) for storage (−20 or 4 °C) prior to dilution in experiments (Karlsson *et al*., 1984; Cerveñansky *et al*., 1994). The perchlorate salt of *N*-methylacridinium was recrystallized ($\epsilon_{358\ nm} = 21\ 300\ M^{-1}\ cm^{-1}$).

Fluorescence Measurements. Data were obtained in a fluorescence temperature jump apparatus similar to that described previously (Rigler *et al*., 1974; Rosenberry & Neumann, 1977). A temperature increase of 2.3 °C with a risetime τ_h of about 12 μs was triggered by a 25-kV discharge from a 21 nF capacitor through a 0.85−1.0-mL sample compartment. The fluorescence trace was initially stored on a Bryans/Physical Data model 523-A transient recorder before transfer to a Hewlett-Packard 9816 computer, where relaxation times and amplitudes were calculated by a modified Marquardt algorithm[1] with nonlinear curve fitting and multiple relaxation analysis capability. According to the time base selected, an instrumental risetime τ_M was imposed[2] and slower chemical relaxations were calculated. Aliquots (5−20 μL) of *N*-methylacridinium were added in four to nine titration steps to AChE in 0.1% Triton X-100, 20 mM sodium phosphate buffer (pH 7.0). Three to six temperature jump measurements were made at each titration step, and intervals of 8−15 min between measurements were imposed to ensure temperature reequilibration. The initial solution was thoroughly deaerated to eliminate bubble formation, but deaeration was not repeated after the titration steps.

Scheme 1



Ligand Interactions with AChE. Scheme 1 represents the kinetic model corresponding to Figure 1 in which fasciculin 2 (**F**) can bind to the AChE (**E**) peripheral site and *N*-methylacridinium (**L**) can bind to the AChE acylation site to form the ternary complex **EFL**. With 20 mM sodium phosphate buffer (pH 7.0) at 25 °C, k_F and k_{-F} for fasciculin 2 binding to human AChE have been estimated as $2.7 \times 10^7\ M^{-1}\ s^{-1}$ and $2.9 \times 10^{-4}\ s^{-1}$, respectively (Eastman *et al*., 1995). These values correspond to an equilibrium dissociation constant $K_F = k_{-F}/k_F$ of 11 pM. In contrast, the equilibrium constant $K_L = k_{21}/k_{12}$ for *N*-methylacridinium binding to human AChE was estimated to be 0.2 μM. Equilibrium constants $K_{F2} = k_{-F2}/k_{F2}$ and $K_L' = k_{21}'/k_{12}'$ for fasciculin 2 and *N*-methylacridinium binding in the ternary complex **EFL**, respectively, were about an order of magnitude higher than the corresponding equilibrium constants for the binding of each ligand to the free enzyme. In experiments that included fasciculin 2 in this report, its concentration always exceeded 4 μM, more than 4 orders of magnitude greater than either K_F or K_{F2}. Thus in these experiments the concentrations of **E** and **EL** became very small and the amplitudes of relaxation reactions involving **E** or **EL** were assumed negligible [*e.g.*, see Thusius (1972)]. In this case the kinetics of *N*-methylacridinium binding to AChE in the presence of saturating concentrations of fasciculin 2 reduces from that in Scheme 1 to that in Scheme 2.

[1] Program written by C.-R. Rabl. Available on request.

[2] The instrumental risetime $\tau_M = (\tau_h^2 + \tau_f^2)^{0.5}$, where τ_h is the risetime of the temperature increase and τ_f is the risetime of electronic filtering imposed to reduce optical noise.

Scheme 2

$$\mathbf{L} + \mathbf{EF} \underset{k_{21}'}{\overset{k_{12}'}{\rightleftharpoons}} \mathbf{EFL}$$

Thermodynamic and Kinetic Measurements. Reactions of *N*-methylacridinium with AChE were monitored by the complete quenching of *N*-methylacridinium fluorescence on binding to AChE (Mooser *et al*., 1972; Rosenberry & Neumann, 1977). Equilibrium titrations of AChE with increasing amounts of *N*-methylacridinium in the absence of fasciculin were analyzed with eq 1,

$$[\mathrm{EL}]\omega = [[\mathrm{L}]_{\mathrm{tot}} - [\mathrm{L}]]\omega = [\mathrm{E}]_{\mathrm{o}}\left[1 + \frac{K_{\mathrm{L}}}{[\mathrm{L}]}\right] \qquad (1)$$

where brackets indicate the concentration of the bracketed species, $[\mathrm{L}]_{\mathrm{tot}} = [\mathrm{L}] + [\mathrm{EL}]$, $[\mathrm{E}]_{\mathrm{o}}$ is the [E] prior to addition of L, and $\omega = (1 + \Delta V/V_{\mathrm{o}})$ is a volume correction in which V_{o} is the initial volume and ΔV is the incremental volume addition of ligand solution to V_{o}. The other parameters are defined in Scheme 1. Since **L** is the only fluorescent species, [L] was directly calculated from the fluorescence by reference to a standard curve with **L** in the absence of **E**. With a saturating concentration of fasciculin 2, eq 1 holds except that **EFL**, **EF**, and K_{L}' replace **EL**, **E**, and K_{L}, respectively.

For the single reversible bimolecular reaction corresponding to Scheme 1 in the absence of fasciculin 2, one relaxation with a rate constant equal to the reciprocal of the relaxation time τ is predicted as given by eq 2 (Eigen & DeMaeyer, 1963).

$$\tau^{-1} = k_{12}[[\mathrm{E}] + [\mathrm{L}]] + k_{21} \qquad (2)$$

The amplitude of this relaxation $\Delta[\mathrm{L}]$ is given by eq 3

$$\Delta[\mathrm{L}] = \left[\frac{[\mathrm{E}][\mathrm{L}]}{[\mathrm{E}] + [\mathrm{L}] + K_{\mathrm{L}}}\right]\left[\frac{\Delta H^{\circ}\,\Delta T}{RT^2}\right] \qquad (3)$$

where ΔH° is the standard reaction enthalpy (difference between products and reactants). The second term on the right of eq 3 is constant for a given temperature change ΔT.

Alternative expressions for eqs 2 (Rosenberry & Neumann, 1977) and 3 that involve only the total concentrations $[\mathrm{L}]_{\mathrm{tot}}$ and $[\mathrm{E}]_{\mathrm{tot}} = [\mathrm{E}] + [\mathrm{EL}]$ are given by eqs 4 and 5, respectively.

$$\tau^{-2} = k_{12}^{\,2}(K_{\mathrm{L}} + [\mathrm{E}]_{\mathrm{tot}})^2 + 2(K_{\mathrm{L}} - [\mathrm{E}]_{\mathrm{tot}})[\mathrm{L}]_{\mathrm{tot}} + [\mathrm{L}]_{\mathrm{tot}}^{\,2} \qquad (4)$$

$$\Delta[\mathrm{L}]/[\mathrm{L}] = \left[\frac{[\mathrm{E}]_{\mathrm{tot}} - [\mathrm{L}]_{\mathrm{tot}} - K_{\mathrm{L}}}{[([\mathrm{E}]_{\mathrm{tot}} + [\mathrm{L}]_{\mathrm{tot}} + K_{\mathrm{L}})^2 - 4[\mathrm{E}]_{\mathrm{tot}}[\mathrm{L}]_{\mathrm{tot}}]^{0.5}} + 1\right]\left[\frac{\Delta H^{\circ}\,\Delta T^{\circ}}{2RT^2}\right] \qquad (5)$$

With a saturating concentration of fasciculin 2 as in Scheme 2, eqs 2−5 hold except that k_{12}', k_{21}', and K_{L}' replace the unprimed constants.

When $[\mathrm{E}]_{\mathrm{tot}}$ is constant during a progressive titration with **L**, $[\mathrm{E}]_{\mathrm{tot}}$ as well as the kinetic and thermodynamic constants in eqs 4 and 5 can be estimated by nonlinear curve fitting as a function of $[\mathrm{L}]_{\mathrm{tot}}$. Slight corrections for enzyme dilution during the titration (Rosenberry & Neumann, 1977) allowed substitution of the initial $[\mathrm{E}]_{\mathrm{tot}} = [\mathrm{E}]_{\mathrm{o}}$ for $[\mathrm{E}]_{\mathrm{tot}}$ in these equations. The corrections assumed that $\Delta V/V_{\mathrm{o}}$ (see eq 1) was both approximately proportional to $[\mathrm{L}]_{\mathrm{tot}}$ and small enough that $(1 + \Delta V/V_{\mathrm{o}})^{-1} = 1 - \Delta V/V_{\mathrm{o}}$ throughout the titration. Then $[\mathrm{E}]_{\mathrm{tot}} = [\mathrm{E}]_{\mathrm{o}}(1 - \rho[\mathrm{L}]_{\mathrm{tot}})$, where $\rho = \Delta V/(V_{\mathrm{o}}[\mathrm{L}]_{\mathrm{tot}})$, and eqs. 4 and 5 were adjusted accordingly. For all titrations in this report $\Delta V/V_{\mathrm{o}}$ was ≤0.13, and the dilution corrections did not alter the estimates of the constant parameters in eqs 4 and 5 by more than 20%.

Means and standard errors of relaxation times and amplitudes for each titration step were used in weighted nonlinear regression analyses (BioSoft program Fig.P, version 6.0) according to eqs 2−5.

Table 1: Thermodynamic and Kinetic Constants for the Interaction of *N*-methylacridinium with AChE and the AChE−Fasciculin 2 Complex

	equilibrium	relaxation amplitude[a]		relaxation time	
temp (°C)	K_{L}[b] (μM)	K_{L} (μM)	ΔH° (kJ/mol)	k_{12} ($\mathrm{nM^{-1}\,s^{-1}}$)	k_{21} ($\mathrm{s^{-1}}$)
			AChE		
25	0.43 ± 0.04	0.42 ± 0.07	−28 ± 5	0.42 ± 0.03[c]	170 ± 40[c]
37	1.0 ± 0.3	1.0 ± 0.2	−46 ± 2	0.75 ± 0.04[d]	750 ± 200[d]
			AChE + Fasciculin 2		
37	4.2 ± 0.7	2.8 ± 0.9	− 12 ± 2	(1.0 ± 0.1) × 10^{-4}[e]	0.40 ± 0.06[e]

[a] Values calculated with eq 5. [b] Values calculated with eq 1. [c] From Figure 3b. [d] From Figure 3c. [e] From a fit of relaxation times τ_4 to eq 2, assuming $K_{\mathrm{L}}' = 4.0 \pm 0.7$ μM.

RESULTS AND DISCUSSION

Thermodynamic and kinetic constants for the interaction of *N*-methylacridinium with AChE were obtained from measurements of the equilibrium fluorescence, relaxation amplitudes, and relaxation times during a progressive titration of the enzyme with increasing concentrations of ligand. Estimates of the total enzyme concentration ($[\mathrm{E}]_{\mathrm{tot}}$) and the equilibrium dissociation constant K_{L} were obtained from the equilibrium fluorescence by fitting the data to eq 1, and the resulting K_{L} values are shown in Table 1.

Relaxations in the Absence of Fasciculin 2. Temperature jump perturbations of the equilibrium resulted in slight increases in K_{L}, and the consequent dissociation of the fluorescent free *N*-methylacridinium ligand from the nonfluorescent complex of the ligand with AChE gave rapid increases in fluorescence like those in Figure 2. Under the conditions in Figure 2a, a prominent relaxation time τ_1 of 158 μs with an amplitude of 10% of the free ligand concentration is the most important feature. A much smaller amplitude relaxation with a τ_2 of 3.5 ms also was detected by the curve-fitting program. The precision with which this minor relaxation could be measured throughout the entire ligand titration was insufficient to permit its modeling as a defined chemical equilibrium. However, τ_2 relaxations in the 1−4-ms range remained a feature of several titration experiments in the absence of fasciculin 2. Their relative amplitudes actually increased to about 35% of those of the more prominent τ_1 at the higher concentrations of *N*-methylacridinium, reducing the precision of the τ_1 estimates.

Analysis of the relaxation amplitudes corresponding to the prominent τ_1 as a function of the total *N*-methylacridinium concentration ($[\mathrm{L}]_{\mathrm{tot}}$) according to eq 5 confirmed that this

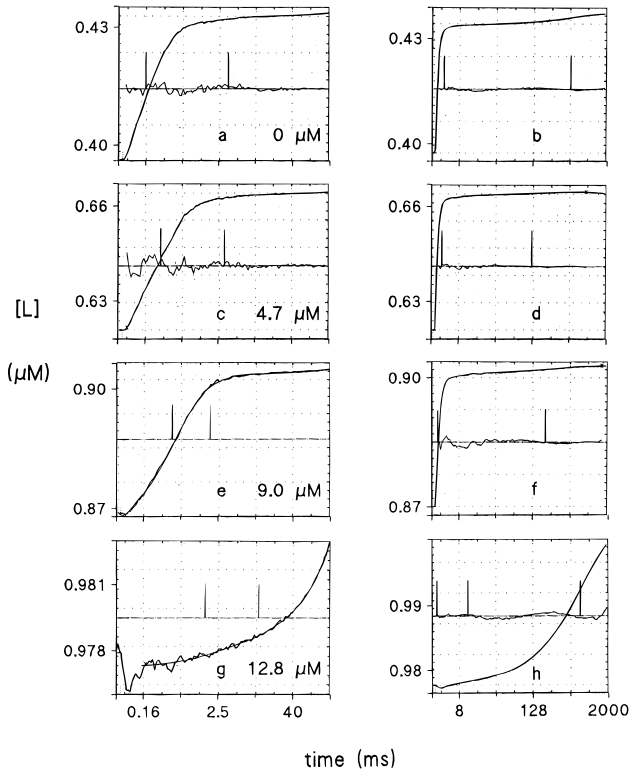


FIGURE 2: Temperature-jump relaxations of *N*-methylacridinium equilibrated with AChE before and after addition of fasciculin 2. A temperature increase of 2.3 degrees (to 310 K or 37 °C) was imposed as described in Experimental Procedures, and measurements for each mixture are shown on two time scales. The initial mixture consisted of 3.2 μM ($[L]_{tot}$) and 11.3 μM ($[E]_{tot}$) in 870 μL (*a* and *b*), and fasciculin 2 was added in 50-μL increments to the concentrations indicated (*c*−*h*). The fluorescence signal has been converted to [L] in μM units, representing unbound *N*-methylacridinium (heavy line in each panel). Data points were collected every 10 μs in the 160-ms traces on the left and every 500 μs in the 2-s traces on the right. The first 16 points in each trace are plotted on a linear time scale, whereas the remaining points are averaged over a logarithmic time scale. Relaxations were determined by a multiexponential fitting program through joint analysis of the fast and slow traces, and relaxation times are indicated by vertical lines on each trace. Instrumental risetimes[2] of 26 μs (left traces), 0.5 ms (*b*, *d*, and *f*), or 1.0 ms (*h*) were deconvoluted in the analysis. In *e* and *g* the fitted curves (thin lines) are plotted over the signal curve, while in the other traces the quality of the fit is indicated by a thin line whose deviation from the horizontal center line represents 5 times the difference between the measured and fitted curves. Observed relaxation times τ_n (with relative amplitudes $\Delta[L]/[L]$ in parentheses) were as follows: *a* and *b*, $\tau_1 = 158$ μs (0.100); $\tau_2 =$ 3.5 ms (0.005); $\tau_4 = 0.52$ s (0.008); *c* and *d*, $\tau_1 = 283$ μs (0.056); $\tau_2 = 3.1$ ms (0.005); $\tau_4 = 0.12$ s (0.002); *e* and *f*, $\tau_1 = 457$ us (0.034); $\tau_2 = 1.8$ ms (0.007); $\tau_4 = 0.2$ s (0.002); *g* and *h*, $\tau_2 = 1.6$ ms (0.0007); $\tau_3 = 11$ ms (0.0009); $\tau_4 = 721$ ms (0.022).

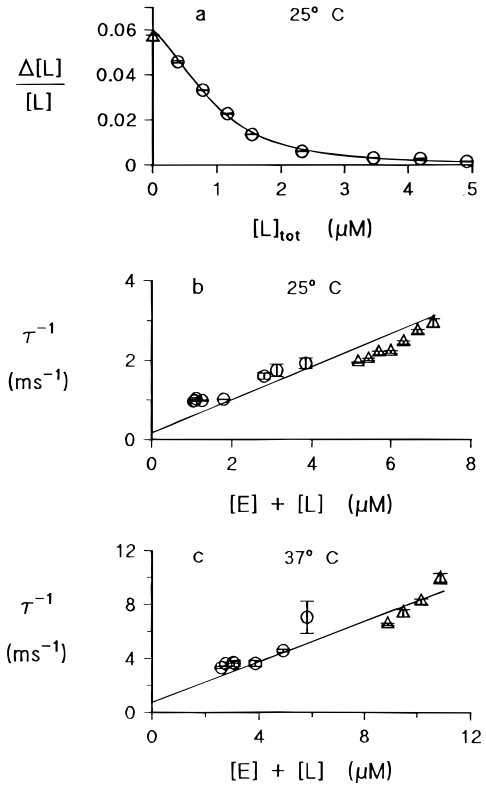


FIGURE 3: Analysis of relaxation data for the binding of *N*-methylacridinium to AChE in the absence of fasciculin 2. The prominent relaxation corresponding to τ_1 was measured as in Figure 2a for a series of $[L]_{tot}$ in two independent titrations (○, △) at 298 K (25 °C) (*a* and *b*) or 310 K (37 °C) (*c*). Concentrations were adjusted such that [L] ≥ [E] for the titrations denoted ○, and [L] ≪ [E] for titrations denoted △. (*a*): Relaxation amplitudes $\Delta[L]$ were analyzed according to eq 5 to give estimates of K_L and $\Delta H°$ listed in Table 1. Since $\Delta[L]/[L]$ was nearly constant for the points denoted △, the $\Delta[L]/[L]$ intercept value from this titration was evaluated separately and adjusted for $[E]_{tot}$ to the value shown. *b* and *c*: Relaxation times τ were analyzed according to eq 2. The analyses assumed fixed values of $K_L = 0.4 \pm 0.1$ μM at 298 K (25 °C) (*b*) or $K_L = 1.0 \pm 0.3$ μM at 310 K (37 °C) (*c*) to obtain the estimates of k_{12} and k_{21} listed in Table 1.

relaxation corresponded to the single equilibrium in Scheme 1 when fasciculin 2 is absent: the independent estimates of K_L obtained from this amplitude analysis agreed well with those from the equilibrium titrations. This is illustrated in Figure 3a, where the $K_L = 0.42$ μM provided by this amplitude analysis at 298 K (25 °C) was within 10% of that determined by the equilibrium titration at about the same temperature (see Table 1). Similar agreement of the K_L value from the analysis of relaxation amplitudes at 37 °C with that from the corresponding equilbrium titration also was observed (Table 1). From these amplitude analyses, estimates of −28 to −46 kJ/mol were obtained for $\Delta H°$ of the equilibrium in Scheme 1 in the absence of fasciculin 2 (Table 1).

The relaxation times τ_1 for combined titration data sets were analyzed according to eq 2. To increase the accuracy of the estimates of k_{12} and k_{21} obtained from these analyses, the weighted linear regressions were fit with the fixed values of K_L obtained from the amplitude analyses and the equilibrium titrations (Figure 3b,c). A k_{12} value of 0.4×10^9 M^{-1} s^{-1} was obtained at 25 °C (Table 1). Although this is a relatively high value for bimolecular reactions of small ligands with proteins, it is about 25% of the k_{12} measured previously at this ionic strength for the association of *N*-methylacridinium with AChE isolated from electric organs of the eel *Electrophorus electricus* (Nolte *et al*., 1980). A k_{21} value of 170 s^{-1} was obtained for human AChE at 25 °C (Table 1) and is comparable to the k_{21} measured previously for the eel enzyme. At 37 °C, the estimated k_{12} increased nearly 2-fold and the k_{21} increased 4−5-fold over the corresponding values for human AChE at 25 °C (Table 1).

Relaxations in the Presence of Fasciculin 2. To demonstrate the incremental effect of fasciculin 2 on the interaction of *N*-methylacridinium with AChE, the toxin was added in three titration steps to the initial mixture in Figure 2a.

Fasciculin 2 suppressed the prominent relaxation corresponding to τ_1 in a progressive fashion. Fasciculin 2 amounts that were substoichiometric with AChE decreased the relative amplitude and increased the τ_1 of this relaxation (Figure 2c,e) until it virtually disappeared with a 20% molar excess of fasciculin 2 (Figure 2g). The changes in amplitude and τ_1 were quantitatively consistent with the k_{12}, k_{21}, and $\Delta H°$ estimates in Table 1.

In addition to these effects, a new range of relaxation times that we denote τ_4 became apparent when AChE was saturated with fasciculin 2. This is shown in Figure 2h, where a τ_4 of 721 ms is the most prominent relaxation detected. A relaxation time this long was technically difficult to measure, as the fluorescence trace became somewhat unstable about 2 s after the temperature jump due to cooling and convection effects. Preliminary experiments suggested that increasing the temperature from 25 to 37 °C decreased the τ_4 values and allowed them to be better resolved from this late region of instability. The amplitude of the prominent τ_4 relaxation increased significantly when the fasciculin 2 concentration exceeded that of AChE (compare Figure 2h with Figure 2b,d,f). Furthermore, with excess fasciculin 2 a much smaller amplitude relaxation denoted τ_3 in the range of 10−200 ms also was detected by the curve-fitting program (see Figure 2h). As with the τ_2 relaxations in the absence of fasciculin 2, the precision with which this minor relaxation could be measured throughout the entire ligand titration was insufficient to permit its modeling as a defined chemical equilibrium. The relative amplitude of τ_3 remained about 5%−15% of that of τ_4 throughout the titration experiment.

To determine whether relaxations corresponding to τ_4 reflected the binding of *N*-methylacridinium to the complex of AChE with fasciculin 2 as defined in Scheme 2, estimates of $[EF]_o$ for the complex and K_L' obtained from analyses of the relaxation amplitudes and times were compared to those from equilibrium titration of the complex with *N*-methylacridinium. In one titration that illustrates these comparisons (Figure 4), estimates of $[EF]_o$ determined independently from the equilibrium binding and the relaxation amplitudes were within 6% of the value expected from the original enzyme activity. The agreement between estimates of K_L' from the equilibrium binding (5.2 μM in Figure 4a) and the relaxation amplitudes (3.9 μM in Figure 4b) also was very good. This quantitative consistency was very important in allowing us to conclude that the very slow relaxations corresponding to τ_4 not only represented a bimolecular reaction like that in Scheme 2 but also that this reaction exhibited the same stoichiometry ($[EF]_o$) and affinity (K_L') observed in the equilibrium titration. When the parameters determined in Figure 4 were combined with those from a second titration of the AChE complex with fasciculin 2 under similar conditions, the averages shown in Table 1 were obtained. An overall average for $K_L' = 4.0$ μM from both the equilibrium binding and relaxation amplitude analyses was then used to fit the relaxation times τ_4 from these titrations to eq 2 to obtain estimates of $k_{12}' = 1.0 \times 10^5$ M^{-1} s^{-1} and $k_{21}' = 0.4$ s^{-1} (Table 1). As suggested by the striking shift to longer relaxation times with saturating fasciculin 2 in Figure 2, these values are more than 3 orders of magnitudes smaller than the corresponding k_{12} and k_{21} values in the absence of fasciculin 2. To obtain a second estimate of k_{12}' independent of any assumptions of $[EL]_o$ and K_L', the relaxation times from the titration in Figure 4 also were analyzed by the second-order analysis in eq 4. This analysis was extremely sensitive to relaxation times at higher $[L]_{tot}$, and its estimates of $[EL]_o$ and K_L' were somewhat lower than those obtained from the equilibrium binding and relaxation amplitude analyses (Figure 4c). Nevertheless, the estimate of $k_{12}' = 1.5 \times 10^5$ M^{-1} s^{-1} from Figure 4c was within 50% of that listed in Table 1. This agreement offers strong support for the striking decreases in k_{12}' and k_{21}' listed in Table 1.

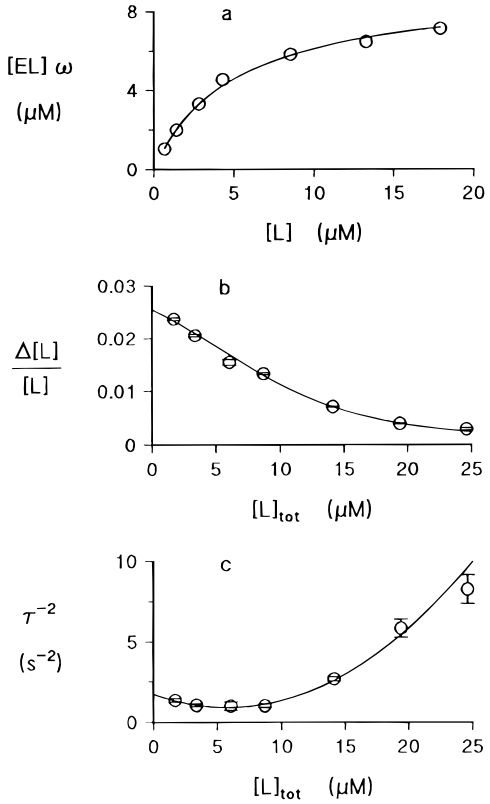


FIGURE 4: Analysis of equilibrium titration and relaxation data for the binding of *N*-methylacridinium to the complex of AChE and fasciculin 2 at 310 K (37 °C). On the basis of prior enzyme activity measurements, the initial concentration of the complex ($[EF]_o$) was 8.9 μM. *a*: Equilibrium titration of the complex with increasing amounts of *N*-methylacridinium was analyzed according to eq 1 with a small correction for the dilution factor ω. Estimates of $[EF]_o = 9.3 \pm 0.3$ μM and $K_L' = 5.2 \pm 0.3$ μM were obtained. *b* and *c*: The prominent relaxation corresponding to τ_4 was measured as in Figure 2h for the series of $[L]_{tot}$ in *a*. Relaxation amplitudes $\Delta[L]$ (*b*) were analyzed according to eq 5 to give estimates of $[EF]_o = 9.4 \pm 0.6$ μM and $K_L' = 3.9 \pm 0.5$ μM. Relaxation times τ (*c*) were analyzed according to eq 4 to give $[EF]_o = 7.2 \pm 0.2$ μM, $K_L' = 1.3 \pm 0.2$ μM, and $k_{12} = (1.5 \pm 0.1)$ x 10^5 M^{-1} s^{-1}.

The three-dimensional structures of fasciculin 2 associated with mouse and torpedo AChEs have recently been determined (Bourne *et al.*, 1995; Harel *et al.*, 1996). It would appear from this structure that access of other ligands to the acylation site through the active site gorge is completely blocked, and the 10^3−10^4-fold decreases in rate constants for *N*-methylacridinium binding to the AChE−fasciculin 2 complex reported here are consistent with this view. Indirect evidence, however, suggests that the extent of this steric blockade may be less with acylation site ligands smaller than the rigid three-ring structure of *N*-methylacridinium. Saturation of the peripheral site with fasciculin 2 resulted in about

[3] J. Yustein and T. L. Rosenberry, unpublished results.
[4] E. W. Eckman and T. L. Rosenberry, unpublished results.

100-fold decreases in k_{cat} for both acetylthiocholine and phenyl acetate (Eastman *et al*., 1995), and the simplest interpretation of the deuterium oxide isotope effects on AChE hydrolysis of these substrates noted in the introduction is that substrate dissociation constants are decreased less than 100-fold. Furthermore, the apparent association rate constant for the transition state analog *m*-trimethylammonium trifluoroacetophenone with the AChE−fasciculin 2 complex is only about 10-fold less than that with the free AChE (Radic *et al*., 1995).[3]

The decrease in k_{cat} for AChE substrates in the AChE−fasciculin 2 complex is just one of several indications that the binding of ligands to the AChE peripheral site results not only in steric blockade but also in a conformational change of the acylation site (Barak *et al*., 1994; Eastman *et al*., 1995; Radic *et al*., 1995). However, the extent of interaction between sites depends on the peripheral site ligand and the substrate. Saturation of this site with propidium resulted in much greater decreases in k_{cat} for acetylthiocholine than for the neutral substrate 7-acetoxy-4-methylcoumarin (Berman and Leonard, 1990) or for phenyl acetate.[4] Covalent conjugation of Pt(terpyridine)Cl with a histidine residue in the peripheral site decreased k_{cat} for acetylcholine to 9% of that with the unmodified AChE control but increased k_{cat} for phenyl acetate to 150% of the control (Haas *et al*., 1992).

The function of the peripheral site in catalytic hydrolysis of the physiological substrate acetylcholine remains to be clarified. One widely considered possibility is that the peripheral site may contribute to an electrostatic field at the active site. Our earlier kinetic studies on eel AChE showed that binding to the active site is controlled by a high net negative charge that can electrostatically attract cationic substrates and inhibitors (Nolte *et al*., 1980), and molecular modeling calculations (Ripoll *et al*., 1993; Antosiewicz *et al*., 1995) from the three-dimensional structure suggest the AChE catalytic subunit has a dipole moment aligned with the active site gorge that can accelerate association rate constants for cationic ligands by more than a factor of 20. However, mutation of up to seven negatively charged residues at or near the gorge rim gave less than a 4-fold decrease in cation association rate constants (Shafferman *et al*., 1994) or calculated dipole moment (Antosiewicz *et al*., 1995), indicating that the peripheral site makes at most a small contribution to the electrostatic field at the active site. Thus additional roles for the peripheral site in the AChE catalytic pathway must be considered. Several years ago it was proposed that the catalytic pathway included an induced-fit conformational change of the initial enzyme−substrate complex (Rosenberry, 1975). With the localization of the peripheral site at the rim of the active site gorge, it now seems plausible to consider whether this site could participate in facilitated delivery of ligands to the acylation site through such an induced-fit conformational change.

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REFERENCES

Antosiewicz, J., McCammon, J. A., Wlodek, S. T., & Gilson, M. K. (1995) *Biochemistry 34*, 4211−4219.
Barak D., Kronman, C., Ordentlich, A., Ariel, N., Bromberg, A., Marcus, D., Lazar, A., Velan, B., & Shafferman, A. (1994) *J. Biol. Chem. 264*, 6296−6305.
Berman, H. A., & Leonard, K. (1990) *Biochemistry 29*, 10640−10649.
Bourne, Y., Taylor, P., & Marchot, P. (1995) *Cell 83*, 503−512.
Cerveñansky, C., Engstrom, A., & Karlsson, E. (1994) *Biochem. Biophys. Acta 1199*, 1−5.
Eastman, J., Wilson, E. J., Cerveñansky, C., & Rosenberry, T. L. (1995) *J. Biol. Chem. 270*, 19694−19701.
Eigen, M., & DeMaeyer, L. (1963) *Tech. Org. Chem. 8* (Part 2), 895−1054.
Haas, R., Adams, E. W., Rosenberry, M. A., & Rosenberry, T. L. (1992) in *Multidisciplinary Approaches to Cholinesterase Functions* (Shafferman, A., & Velan, B., Eds.) pp 131−139, Plenum Press, New York.
Harel, M., Schalk, I., Ehret-Sabatier, L., Bouet, F., Goeldner, M., Hirth, C., Axelsen, P. H., Silman, I., & Sussman, J. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9031−9035.
Harel, M., Kleywegt, G., Ravelli, R., Silman, I., & Sussman, J. L. (1996) *Structure* (in press).
Karlsson, E., Mbugua, P. M., & Rodriguez-Ithurralde, D. (1984) *J. Physiol. (Paris) 79*, 232−240.
le Du, J. H., Marchot, P., Bougis, P. E., & Fontecilla-Camps, J. C. (1992) *J. Biol. Chem. 267*, 22122−22130.
Marchot, P., Khelif, A., Ji, Y.-H., Mansuelle, P., & Bougis, P. E. (1993) *J. Biol. Chem. 268*, 12458−12467.
Mooser, G., Schulman, H., & Sigman, D. S. (1972) *Biochemistry 11*, 1595−1602.
Nolte, H.-J., Rosenberry, T. L., & Neumann, E. (1980) *Biochemistry 19*, 3705−3711.
Radic, Z., Pickering, N. A., Vellom, D. C., Camp, S., & Taylor, P. (1993) *Biochemistry 32*, 12074−12084.
Radic, Z., Duran, R., Vellom, D. C., Li, Y., Cerveñansky, C., & Taylor, P. (1994) *J. Biol. Chem. 269*, 11233−11239.
Radic, Z., Quinn, D. M., Vellom, D. C., Camp., S., & Taylor, P. (1995) *J. Biol. Chem. 270*, 20391−20399.
Rigler, R., Rabl, C.-R., & Jovin, T. M. (1974) *Rev. Sci. Instrum. 45*, 580−588.
Ripoll, D. R., Faerman, C. H., Axelsen, P. H., Silman, I., & Sussman, J. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5128−5132.
Roberts, W. L., Kim, B. H., & Rosenberry, T. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7817−7821.
Rosenberry, T. L. (1975) *Adv. Enzymol. 43*, 103−218.
Rosenberry, T. L., & Neumann, E. (1977) *Biochemistry 16*, 3870−3878.
Rosenberry, T. L., & Scoggin, D. M. (1984) *J. Biol. Chem. 259*, 5643−5652.
Schalk, I., Ehret-Sabatier, L., Bouet, F., Goeldner, M., & Hirth, C. (1992) in *Multidisciplinary Approaches to Cholinesterase Functions* (Shafferman, A., & Velan, B., Eds.) pp 117−120, Plenum Press, New York.
Shafferman, A., Ordentlich, A., Barak, D., Kronman, C., Ber, R., Bino, T., Ariel, N., Osman, R., & Velan, B. (1994) *EMBO J. 13*, 3448−3455.
Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., & Silman, I. (1991) *Science 253*, 872 - 879.
Taylor, P., & Lappi, S. (1975). *Biochemistry 14*, 1989−1997.
Thusius, D. (1972) *J. Am. Chem. Soc. 94*, 356−363.
Weise, C., Kreienkamp, H.-J., Raba, R., Pedak, A., Aaviksaar, A., & Hucho, F. (1990) *EMBO J. 9*, 3885−3888.

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