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Tacrine Protection of Acetylcholinesterase from Inactivation by Diisopropylfluorophosphate: A Circular Dichroism Study

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SUMMARY

Tacrine (1,2,3,4-tetrahydro-9-aminoacridine) showed an apparent noncompetitive inhibition of *Torpedo* acetylcholinesterase (AChE) with a dissociation constant, K_I, of 8.5 nM. It altered the CD bands of AChE in the near-UV region, which monitor the local conformation of aromatic side groups, but not those in the far-UV region, which measure the secondary structure. An extrinsic CD band was induced at 348 nm, with a molar ellipticity of 35,000 deg cm^2 $dmol^{-1}$ (bases on tacrine), when each AChE subunit (M_r = 67,000) was saturated with one tacrine (mol/mol). With this band as a probe, the bound tacrine could be displaced by edrophonium or decamethonium, both of which are known to bind to the anionic site at the active center of AChE, but not by propidium, which binds to the peripheral site of the enzyme. Tacrine protected AChE from inactivation by diisopropylfluorophosphate (DFP). AChE completely lost its enzymatic activity when 1 mol of DFP was bound per mol of subunit upon incubation of 7 μM AChE (subunit) with 100 μM DFP for 40 min, but tacrine-treated AChE retained 60% of its activity and bound only 0.2 mol of DFP per mol of subunit under similar conditions. The corresponding CD, at 348 nm, of the AChE-tacrine-DFP complex increased or decreased gradually, depending on the order of addition of tacrine and DFP, and reached an equilibrium value (80% of its original) after 2 days. The difference absorption spectrum of the AChE-tacrine-DFP complex was the same as that of the AChE-tacrine complex. These results suggest that the protective effect of tacrine may be due to steric hindrance at the esteratic site of the enzyme.

AChE (acetylcholine acetylhydrolase, EC 3.1.1.7) functions in the transmission of nerve-nerve and neuromuscular synapses by hydrolyzing ACh to terminate the ACh receptor-mediated ion gating. The enzyme can be inhibited by many structurally diverse drugs, pharmacological agents, and organophosphorus insecticides and nerve agents, which bind to one or two of the three distinctive sites of the enzyme, namely, esteratic (site 1) and anionic (site 2) or hydrophobic (site 2′), at the active center, and peripheral anionic (site 3) sites (1). Because the substrate ACh binds to sites 1 and 2 simultaneously, in theory reversible ligands that bind to site 1 or 2 directly compete with the substrate and would inhibit the enzyme competitively, whereas those binding to site 3 induce an allosteric effect on site 1 (2–4) and would inhibit the enzyme noncompetitively. However, in practice, most AChE ligands show a mixed-type inhibition (5–7).

Organophosphorus compounds such as DFP and pinacolylmethylphosphonofluoridate (Soman) irreversibly inhibit AChE by phosphorylating the Ser residue at the active center, but they can be dephosphorylated by aldoximes such as 2-PAM. However, prolonged reaction of the enzyme with these inhibitors leads to aging of the phosphorylated AChE that involves dealkylation of the inhibitor molecule and the aged AChE can no longer be dephosphorylated by 2-PAM (8–11). Thus, a general effort for research in this area has been to understand the mechanism of ligand binding and aging of the inactivated AChE and to develop effective antidotes for treating organophosphate poisoning.

1,2,3,4-Tetrahydro-9-aminoacridine (tacrine) was first synthesized by Albert and Gledhill (12) as an antibacterial agent and later found to have anti-AChE activity (13, 14). Tacrine has since been used as a decurarizing agent (15) and to treat intoxications with psychotropic drugs known to have strong anticholinergic effect (16). Recently, tacrine was reported to effectively restore memories of patients with Alzheimer's disease (17). Because of this renewed interest in its medical use, it is important to understand the fundamental mechanism of tacrine inhibition of AChE on a molecular basis. The biochemical aspects of tacrine (18, 19), as well as its protective effect on AChE against organophosphorus inhibitors (20), have been reported, but there was a large discrepancy in the reported

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ABBREVIATIONS: AChE, acetylcholinesterase; DFP, diisopropylfluorophosphate; Soman, pinacolylmethylphosphonofluoridate; 2-PAM, 2-(hydroxyliminomethyl)pyridinium; ACh, acetylcholine.

affinity of tacrine toward AChE [the dissociation constant, K_I, was 5 nM (18) or 2 μM (19)]. These studies were done with partially purified AChE (18, 19) or with brain homogenate (20). It is therefore worthwhile to reinvestigate the interaction of tacrine with highly purified AChE and the mechanism of its protective effect against DFP. We used CD as a probe in this report. Tacrine bound to AChE induced an extrinsic CD band in the near-UV region, which can monitor any conformational change of the AChE-tacrine complex upon binding of DFP. We will present evidence that tacrine bound to the active center of AChE at either the anionic or hydrophobic site and protected the enzyme from inactivation by DFP at the esteratic site through steric hindrance.

Experimental Procedures

Materials. The tetramer of AChE (G_4, 11 S) was extracted from *Torpedo californica* and purified on an acridine affinity column (21). Unless stated otherwise, the protein solution contained 0.1 M NaCl, 10 mM Tris·HCl, and 10 mM $MgCl_2$ at pH 7.5. Protein concentrations were determined by the Lowry method, using serum γ-globulin as a standard. They were expressed in μM for the subunit of AChE (M_r = 67,000). Enzyme activities were measured by the method of Ellman *et al.* (22) at room temperature (22–23°) in a 2.5-ml solution containing 0.1 M sodium phosphate, 5 mM $MgCl_2$, 57 μM acetylthiocholine, and 3.8 mM 5,5′-dithiobis-(2-nitrobenzoic) acid. A Zeiss PMQ II spectrophotometer with a recorder was used to measure the absorbances at 412 nm monitored in this method. All spectroscopic properties were determined in a buffer consisting of 0.1 M NaCl, 10 mM Tris·HCl, and 10 mM $MgCl_2$ (pH 7.5).

Tacrine (Aldrich Chemical Co., Milwaukee, WI) and DFP (Sigma Chemical Co., St. Louis, MO) were used without further purification, as were decamethonium chloride, edrophonium chloride, and propidium iodide (all from Sigma). [^{3}H]DFP (New England Nuclear, Boston, MA) showed a single peak upon gel filtration on a Bio-Gel P-2 (Bio-Rad, Richmond, CA) column, indicating that the product was pure. Soman was supplied by the United States Army Medical Research Institute of Chemical Defense. Whatman DE81 ion-exchange discs were obtained from Chemtrix.

Enzyme kinetics. Irreversible inactivation of AChE by DFP (in the presence of tacrine) follows a first-order reaction and its rate constant, k_i, is determined from (23)

$$k_i = 2.303 \log(v_1/v_2)/(t_2 - t_1)[I_{ir}] \quad (1)$$

Here v_1 and v_2 are the rates of hydrolysis of the substrate at times t_1 and t_2, respectively, and $[I_{ir}]$ is the concentration of the irreversible inhibitor. Experimentally, 500 μl of 20 nM AChE were incubated with 0 to 2 μM tacrine (a reversible inhibitor) for 5 to 10 min; 19 μM DFP was then added and 50-μl aliquots of the incubation mixture were withdrawn at intervals for the assay of the enzyme activity by the method of Ellman *et al.* (22).

Reversible inhibition of enzymes is tradditionally represented by

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightleftharpoons} E + P \quad (2)$$

$$E + I \overset{K_I}{\rightleftharpoons} EI \quad (3)$$

$$ES + I \overset{K_I^S}{\rightleftharpoons} ESI \quad (4)$$

$$EI + S \overset{K_S^I}{\rightleftharpoons} EIS \quad (5)$$

where E, S, and I are the enzyme, substrate, and reversible inhibitor, respectively. K_I, K_I^S, and K_S^I are the dissociation constants of the complexes EI, ESI, and EIS. The data can be graphically represented by the Lineweaver-Burk plot of $1/v$ versus $1/[S]$ (24), v being the initial rate of an enzyme reaction. From the slopes and intercepts of the double reciprocal plots, the inhibitor can be identified as competitive, noncompetitive, uncompetitive, or mixed.

Binding isotherm. The binding of [^{3}H]DFP to AChE was performed at room temperature by incubating 2 μM (subunit) AChE with or without 10 μM tacrine for 5 min. DFP (100 μM) and an appropriate volume of buffer (0.1 M NaCl, 10 mM Tris·HCl, and 10 mM $MgCl_2$, pH 7.5) were added to the solution, to a total volume of 500 μl. At 10-min intervals, 20-μl aliquots were spotted in duplicate on a Whatman DE81 ion-exchange disc and washed twice in 200 ml each of 20 mM sodium phosphate buffer (pH 7.5) in separate beakers. The discs were counted in a Beckman LS 150 scintillation counter. Discs without washing gave the total count, and those with washing for a mixture without enzyme served as the blank. The enzymatic activities of the incubation mixture were measured at intervals by diluting 4 μl of the mixture with 200 μl of 0.1 M sodium phosphate and 5 mM $MgCl_2$ (pH 7.5). Ten microliters of the diluted mixture were used in the Ellman assay.

CD. CD spectra were measured on a Jasco J-500A spectropolarimeter equipped with a DP-500 data processor for data acquisition and an IBM personal computer for data analysis. The temperature of the protein solution was maintained at 25° with a water-jacketed cell holder attached to a Haake constant-temperature bath. The data below 310 nm were expressed as mean residue ellipticity, [Θ], in deg cm^2 dmol^{-1}; $[\Theta] = M_0\psi/lc$, where ψ is the ellipticity in mdeg, M_0 the mean residue weight (114 for AChE), l the cell path length in mm, and c the protein concentration in mg ml^{-1}. The data above 310 nm were expressed as molar ellipticity, [Θ], in deg cm^2 dmol^{-1}, based on the concentration of bound tacrine. When AChE is saturated with tacrine, the molar concentration of bound tacrine is equivalent to that of the AChE subunit; thus, $[\Theta] = (67{,}000/114)[\theta]$. The noise of the CD spectra for AChE solutions was exceedingly low at wavelengths above 200 nm. The signal-to-noise ratio in the 190-nm region was around 5 for manual scanning and greater than 20 for computer-averaged multiple scanning (four or more).

Absorption spectroscopy. Absorption spectra of AChE solutions were measured on a Cary 118 recording spectrophotometer at room temperature. For difference absorption spectra, tandem cells of 0.44-cm path length were used. The reference cell contained unmixed solutions in separate compartments and the sample cell, the mixed solution distributed equally in both compartments.

Results

Inhibition kinetics of AChE by tacrine. The inhibition of AChE by tacrine was reversible. The bound tacrine could be removed through prolonged dialysis or by dilution, as judged by the disappearance of an induced CD band at 348 nm (to be described later) and the recovery of the enzyme activity. The double-reciprocal plots of the enzyme kinetic data in the presence of tacrine showed a family of straight lines with a common intercept on the abscissa (Fig. 1). According to the traditional treatment, tacrine appeared to be a noncompetitive inhibitor, which reacts with both the enzyme (Eq. 3) and the ES complex (Eq. 4). Assuming $K_I = K_I^S$ and $K_S^I = k_1/k_{-1}$, Eqs. 2–5 lead to

$$1/v = (K_M/V)(1 + [I]/K_I)/[S] + (1 + [I]/K_I)/V \quad (6)$$

where v and V are the initial and maximum rates of the enzyme reaction, $[I]$ and $[S]$ are the concentrations of inhibitor and substrate, and K_M is the Michaelis-Menten constant. We obtained a K_I value of 8.5 nM for tacrine, which was comparable to 5 nM as reported by Heilbronn (18) but considerably smaller than 2.2 μM reported by Patocka *et al.* (19), both for human erythrocyte. Our K_I value was also much smaller than the dissociation constant of edrophonium (250 nM), decamethonium (1.5 μM), and propidium (300 nM) (25) and that of *N*-methylacridinium (230 nM), from fluorescence titration (6, 7).

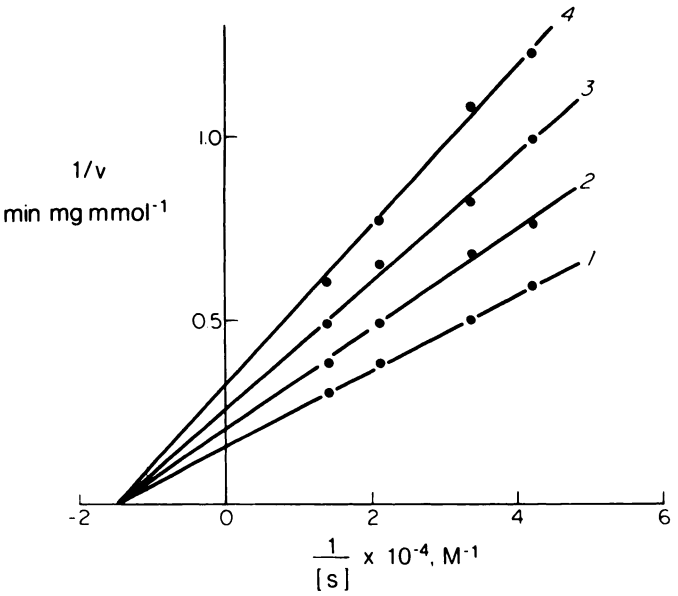

Fig. 1. Double-reciprocal plots of the initial rate of reaction versus substrate concentration in the presence of tacrine. Concentrations of tacrine: *1*, 0; 2, 3.97 nM; *3*, 7.91 nM; *4*, 11.9 nM. The enzyme activity was measured by the method of Ellman *et al.* (22) using acetylthiocholine as the substrate.

Thus, the binding affinity of tacrine toward AChE was much higher than that of the other ligands.

Accumulating evidence (5, 26) now suggests that AChE and its substrate form at least two intermediates, *ES* and its acylenzyme *ES'*. Thus, the traditional treatment by Eqs. 2–5 may be oversimplified and the observed noncompetitive inhibition of AChE by many ligands is only apparent (see Discussion).

Absorption spectrum of tacrine. Tacrine is a pale yellow powder and its dilute aqueous solution was almost colorless unlike its analog 9-aminoacridine, which is deep yellow and fluorescent. The absorption spectrum of tacrine in water showed two maxima at 324 and 336 nm, with molar absorption coefficients of 1.23×10^4 and 1.09×10^4 M^{-1}, respectively (Fig. 2). There were two or three small overlapping peaks on the shoulder of the shorter wavelength side of the 324-nm band, and another strong band below 250 nm.

Effect of tacrine on CD spectrum of AChE. The CD spectrum of AChE between 190 and 250 nm showed a double minimum of 222 and 208 nm and a maximum at 192 nm (Fig. 3, *curve 1*), which are characteristic of the presence of α-helix. Analysis of the data by a least-squares method (27, 28) indicated a moderate amount of α-helix (40%) and β-sheet (35%) and a trace of β-turn (21). The addition of tacrine did not alter the CD spectrum in this region (Fig. 3, *curve 2*), suggesting that the binding of tacrine did not change the secondary structure of the enzyme.

The CD spectrum of AChE in the near-UV region, which monitors the local conformation of aromatic side groups (Fig. 4, *curve 1*), was affected by the addition of tacrine (Fig. 4, *curve 2*). A positive band appeared at 256 nm and the negative 276-nm band almost disappeared. The bands at 286 and 294 nm became more negative than those of AChE without tacrine. In addition, bound tacrine induced an extrinsic positive band at 348 nm, which can be used as a probe for the binding of tacrine to AChE. The molar ellipticity, $[\Theta]_{348}$, based on the concentration of bound tacrine, was 35,000 deg cm^2 dmol^{-1} when the

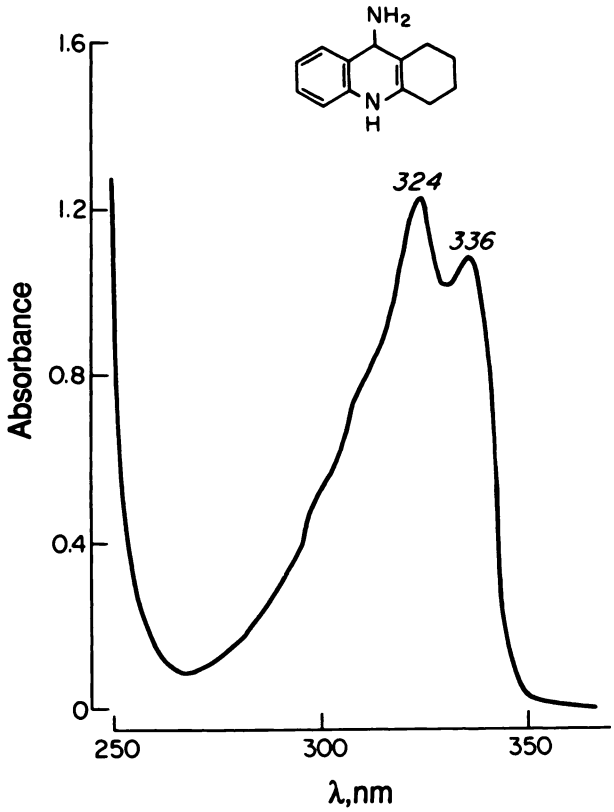

Fig. 2. Absorption spectrum of tacrine in water at 23°. Concentration: 100 μM in 1-cm cell.

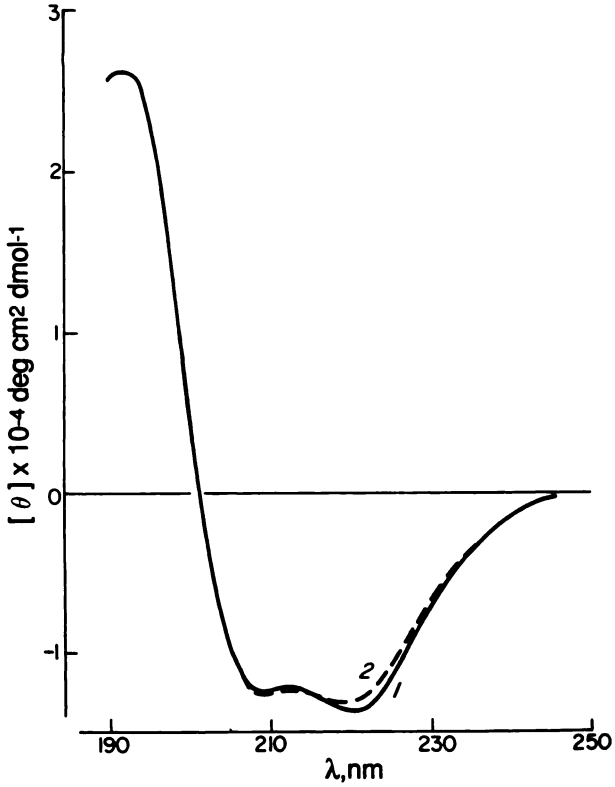

Fig. 3. Far-UV CD spectra of AChE at 25°. Concentration of AChE: 0.34 mg/ml (or 5.1 μM) in buffer (0.1 M NaCl, 10 mM Tris·HCl, and 10 mM $MgCl_2$, pH 7.5). *Curves*: *1*, with no tacrine; 2, with 132 μM tacrine.

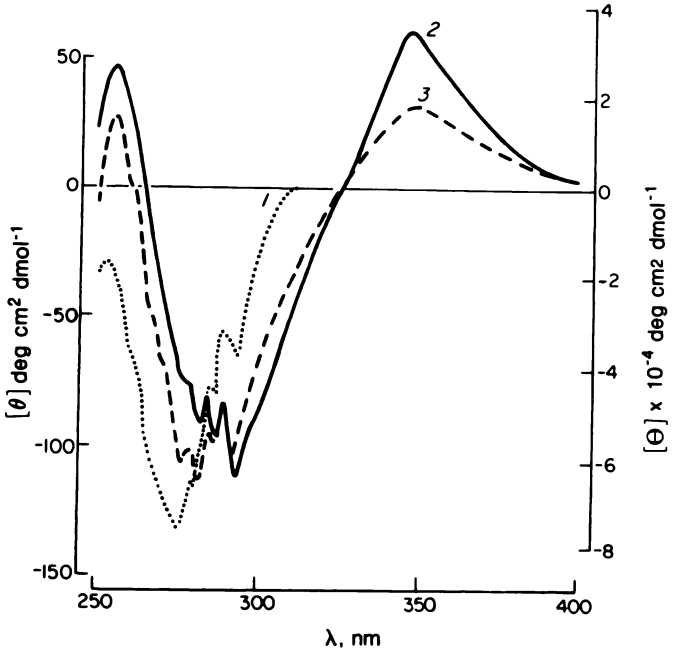

Fig. 4. Effect of tacrine and DFP on the near-UV CD spectra of AChE at 25°. *Curve 1*, 5.1 μM AChE; *curve 2*, 5.1 μM AChE plus 130 μM tacrine; *curve 3*, 5.1 μM AChE plus 130 μM tacrine and 100 μM DFP. DFP was added to the AChE-tacrine complex from *curve 2*. The mixture was incubated for 3 hr at room temperature before the CD spectrum was recorded.

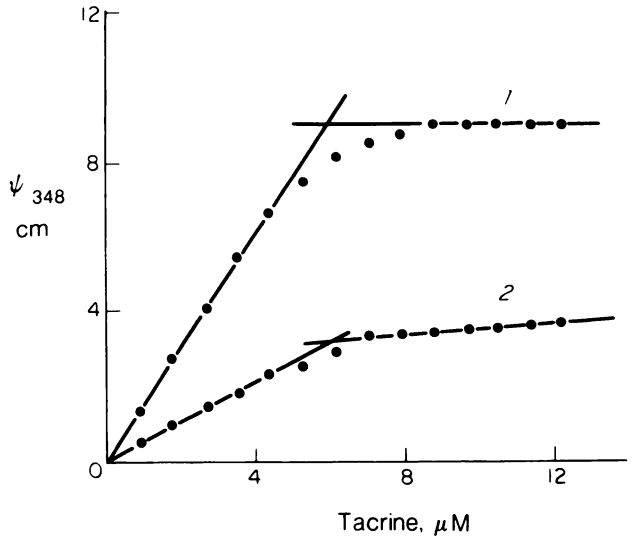

Fig. 5. Titration of AChE with tacrine based on CD at 348 nm. *Curve 1*, 3 ml of 5.1 μM AChE in 1-cm cuvette were titrated with 0.5 mM tacrine. *Curve 2*, concentrated DFP was added to 3 ml of AChE, to 100 μM, and, after 1-hr incubation, it was titrated with tacrine as in *curve 1*. The small increase in the ellipticity above endpoint was due to a slow change of the AChE-tacrine-DFP complex as shown in Fig. 6.

binding site of AChE was saturated with tacrine. The spectra of the AChE-tacrine complex in the presence of excess tacrine (12 or 130 μM) were essentially identical. Tacrine itself had no CD signals in water and was included in the blanks.

The addition of DFP to the AChE-tacrine complex did not change the far-UV CD spectrum of the complex, but it seemed to shift its near-UV spectrum toward that of native enzyme (Fig. 4, *curve 3*). The magnitudes of the CD bands at 256 and 294 nm became smaller and those at 276 and 284 nm larger than the magnitudes for the AChE-tacrine complex. The spectrum of the ternary complex was intermediate between that of native AChE and the tacrine-modified enzyme. Because the addition of DFP alone did not alter the CD spectrum of AChE in both the far- and near-UV regions (not shown), the observed changes in Fig. 4 were probably due to the orientation of the bound tacrine on the enzyme. This was accompanied by a reduction in the magnitude of the tacrine-induced band at 348 nm. The spectrum shifted back slightly toward that of AChE-tacrine complex after 2 days at room temperature.

Titration of AChE with tacrine, based on the ellipticities at 348 nm, indicated that exactly 1 mol of tacrine bound to 1 mol of the AChE subunit (Fig. 5, *curve 1*). The rather sharp endpoint suggested a strong binding of tacrine to AChE. The dissociation constant, K_I, could not be determined by the CD method because concentrations of both reactants higher than K_I were required for accurate CD signals. DFP-inactivated AChE gave the same endpoint as native enzymes, that is, 1 mol of tacrine/mol of AChE subunit (Fig. 5, *curve 2*). However, the magnitude of $[\theta]_{348}$ was smaller for the phosphorylated AChE than for the native enzyme, suggesting that the orientation of the bound tacrine molecule might be different in the presence and absence of DFP. Because DFP is covalently bound to the estratic site of AChE (9), tacrine may be bound to a site distinct from the DFP-bound site.

Time dependence of CD of the AChE-tacrine-DFP complex. The magnitude of the tacrine-induced CD band at 348 nm decreased (from 100%) or increased (from 0%) with time, depending on whether DFP was added before or after tacrine to the AChE solution. The magnitude of CD approached a plateau after prolonged reaction, regardless of the order of addition of the ligands. For DFP (100 μM)-inactived AChE, $[\Theta]_{348}$ rose immediately from 0 to 27% upon addition of tacrine to the AChE-DFP complex, then gradually to 55% after 7 hr (Fig. 6B, *curves 1* and *3*) and to 82% of that of the AChE-tacrine complex after 2 days (Fig. 6A, *curves 1* and *3*). If DFP was added after AChE was reacted with tacrine, $[\Theta]_{348}$ decreased from 100% to about 75% after 8 hr (Fig. 6B, *curve 2*) and approached 82% after 2 days (Fig. 6A, *curve 2*). Inactivation of AChE by DFP for 1 hr (*curve 1*) and 20 hr (*curve 3*) showed essentially the same time dependence of CD upon addition of tacrine, indicating that aging of the AChE-DFP complex was not responsible for the slow change in $[\Theta]_{348}$ with time. The rate of change in the induced CD could be increased by raising the concentration of DFP; for instance, with 1 mM instead of 100 μM as used in the experiments shown in Fig. 6, the time to reach equilibrium was shortened to less than 1 day (not shown).

In a separate experiment, we substituted another organophosphorus compound, 15 μM Soman (a potent nerve agent), for DFP and repeated the CD study of the ternary complex. The trend of the CD change was the same as for DFP, but the time required for attaining an equilibrium value of $[\Theta]_{348}$ was shortened to within 30 min instead of 2 days, regardless of whether tacrine or Soman was first reacted with AChE.

Protective effect of tacrine against AChE inactivation by DFP. Although it was an apparent noncompetitive inhibitor, tacrine could protect AChE from inactivation by DFP (Fig. 7A). In the absence of tacrine, the enzymatic activity of AChE decreased with increasing time, when 7.2 μM AChE was incubated with 100 μM DFP, and dropped to 0 in about 80 min.

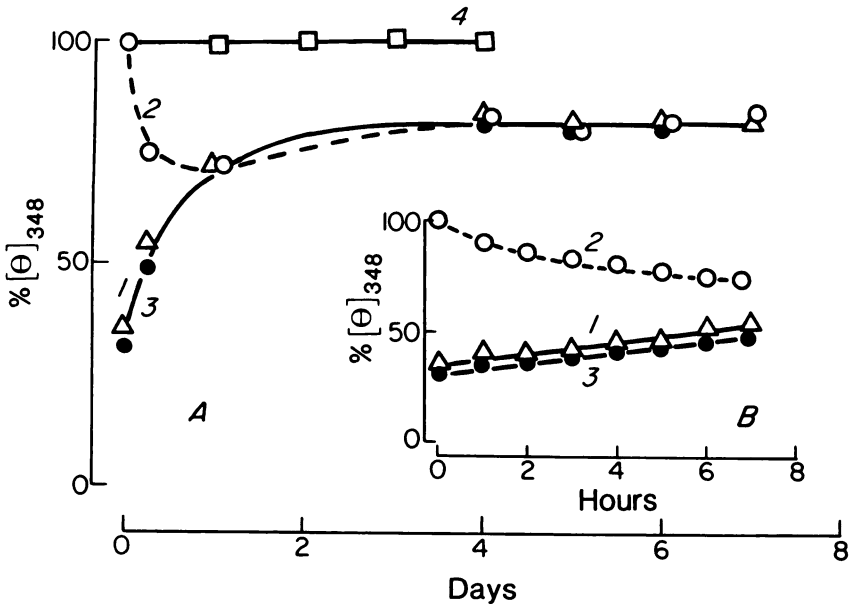

Fig. 6. Time dependence of $[\Theta]_{348}$ of the AChE-tacrine-DFP complex at 25°. *Curve 1*, AChE reacted with DFP for 1 hr and then with tacrine; *curve 2*, AChE reacted with tacrine for 10 min and then with DFP; *curve 3*, AChE reacted with DFP for 20 hr (aged) and then with tacrine; *curve 4*, AChE plus tacrine as the control. Concentration: AChE, 0.36 mg/ml (or 5.4 μM); DFP, 100 μM; tacrine, 18 μM.

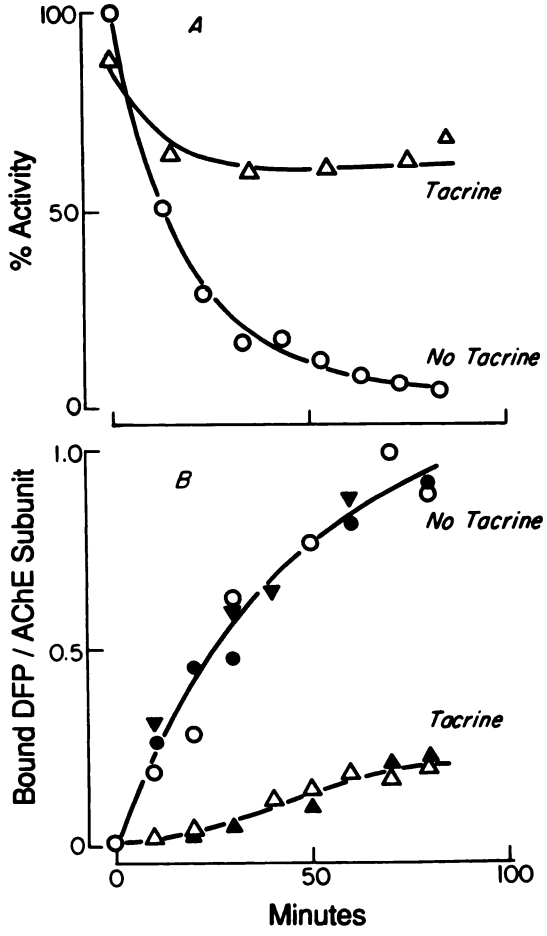

Fig. 7. Effect of tacrine on the AChE activity and binding of DFP by AChE. A 500-μl solution of 0.48 mg/ml (or 7.2 μM) AChE and 100 μM [^{3}H]DFP with or without 18 μM tacrine was incubated at room temperature. Aliquots were withdrawn at intervals for the assay of enzymatic activity (A) and radioactivity counts (B).

However, in the presence of 18 μM tacrine, the activity of AChE decreased slowly from 86% of its original value to 60% at 80 min (Fig. 7A) and approached 0 after 2 days (not shown). For the assay of enzyme activity, the incubation mixture was diluted by more than 1000 times (see Experimental Procedures); thus, a large fraction of bound tacrine would have dissociated from the enzyme and resulted in only 10–15% inhibition at 0 times (Fig. 7A, *triangles*). As the reaction between AChE and DFP was also stopped by dilution of the incubation mixture, the observed activity curve actually showed a protective effect of tacrine during incubation against DFP. (Under identical experimental conditions, the relative activities of AChE in the absence and presence of tacrine should not be complicated by the decrease in free DFP concentration due to hydrolysis.) The rate of inactivation of tacrine-bound AChE by DFP paralleled the change in $[\Theta]_{348}$ with time (cf. Fig. 6B, *curve 2*). Further, the rate of inactivation was proportional to the concentration of added DFP (not shown).

Concomitantly, the binding study of [^{3}H]DFP to AChE showed that the addition of tacrine could hinder the irreversible binding of DFP to the enzyme (Fig. 7B). In the absence of tacrine, 1 mol of DFP bound to 1 mol of the AChE subunit after reaction with 100 μM DFP for 80 min. However, if the enzyme was first reacted with tacrine, only 0.2 mol of DFP/mol of subunit was bound to the enzyme during the same period. The degree of inhibition for the binding of DFP roughly paralleled the degree of protection of activity as shown above. Additional binding, if any, beyond 80 min was not measured because dealkylation of the bound DFP might introduce some uncertainties (both isopropyl groups on DFP were labeled). These results suggest that the binding of tacrine to AChE prevented DFP from approaching the catalytic site of the enzyme.

The protective effect of tacrine was further demonstrated by the bimolecular rate constant of inactivation as determined from Eq. 1. The k_i value was 1.4×10^4 M^{-1} min^{-1} in the absence of tacrine, decreased with increasing concentration of tacrine, and reached a plateau of 70 M^{-1} min^{-1} above 1 μM tacrine, probably because of saturation of the bound tacrine on AChE.

Difference absorption spectrum of the AChE-tacrine complex in the presence and absence of DFP. In spite of their differences in the CD spectra in the near-UV region (Fig. 4), the AChE-tacrine and AChE-tacrine-DFP complexes after 24-hr incubation had essentially the same difference absorption spectrum (Fig. 8), indicating that tacrine bound to the enzyme was probably not displaced by the addition of DFP. Both spectra showed two negative bands at 324 and 336 nm, with a shoulder near 310 nm. The positive absorption band at 348 nm corresponded to the induced CD band of tacrine at the same wavelength; their molar difference absorbances were identical.

Displacement of bound tacrine by other ligands. Like tacrine, the ligands edrophonium, propidium, and decamethonium bind to AChE. They do not have CD bands above 300 nm, either in the free or bound state. Thus, any release of tacrine from AChE by these competing ligands can be monitored by the reduction in magnitude of the 348-nm band for bound tacrine (Fig. 9). Edrophonium is known to bind to the anionic site (site 2) at the active center, through its cationic group, and its phenolic group is hydrogen-bonded to another residue at the active site (25, 29). This ligand gradually displaced bound tacrine (11 μM) with increasing concentration of edrophonium and approached a plateau of 80% displacement at 700 μM edrophonium. All bound tacrine could be released upon addition of excess edrophonium, e.g., 10 mM. Propidium binds to the peripheral anionic site (site 3) (25, 30). Unexpectedly, this ligand could also displace about 15% of the bound tacrine when 10–200 μM propidium was added to the AChE-tacrine complex, but the intensity of the tacrine-induced CD band reversed to its original value as the propidium concentration was raised to 10 mM. The initial reduction in $[\Theta]_{348}$ was probably due to an allosteric effect of bound propidium at the peripheral site (25, 30), but the reversal of the CD value to its original one at high concentrations of propidium is not yet understood. Decamethonium, a bifunctional ligand that binds to both the peripheral (site 3) and anionic (site 2) sites (7, 31), initially displaced the bound tacrine more than edrophonium at concentrations below 100 μM, but only 60% at 700 μM and 80% at 10 mM decamethonium.

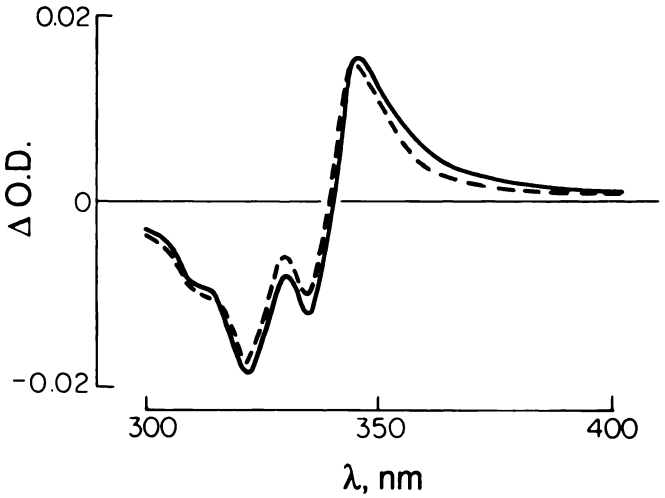

Fig. 8. Difference absorption spectra of the AChE-tacrine and AChE-tacrine-DFP complexes at room temperature. For the AChE-tacrine complex (– – –), 10.8 μM AChE and 36 μM tacrine were filled separately into the two compartments of a tandem cell (reference) and equal volumes of the two solutions were mixed and filled into the two compartments of another tandem cell (sample). For the AChE-tacrine-DFP complex (——), the sample from *curve 2* of Fig. 6 after 1 day of incubation was used. The reference cell contained the same solutions as in the AChE-tacrine complex, except that 200 μM DFP was added to the compartment containing tacrine. The spectrum was unaffected by the hydrolysis of free DFP, which does not absorb in the near-UV region.

Discussion

Enzyme kinetics. In the traditinal treatment of reversible inhibition of enzymes, the enzyme (E) and substrate (S) form a steady state intermediate (ES) (Eq. 2). A competitive inhibitor, which binds with the enzyme to form an EI complex (Eq. 3), increases the Michaelis-Menten K_M without affecting the maximum velocity, V, and an uncompetitive inhibitor, which binds only with the ES complex (Eq. 4), decreases both the K_M and V values equally, whereas a noncompetitive inhibitor, which binds with both E and ES (Eqs. 2–5), decreases the V value without affecting the K_M value. In addition, there are mixed-type inhibitions, which vary both the K_M and V values unequally. Experimentally, the Lineweaver-Burk plots of $1/v$ versus $1/[S]$ (23) yield a common intercept on the ordinate, $(1/v)$ axis, for competitive inhibition, a common intercept on the abscissa, $(1/[S])$ axis, for noncompetitive inhibition, and a series of parallel lines for uncompetitive inhibition. It can be shown that the noncompetitive effect is just the sum of the competitive and uncompetitive effects according to the traditional theory. Our results in Fig. 1 appeared to show that tacrine was a noncompetitive inhibitor for AChE. However, this does not imply that true noncompetitive inhibition occurs only when there is combination with two forms. Often, many mecanisms can give the same inhibitory effects. There is no unequivocal mechanism for the observed kinetic data. Rather, they can eliminate those mechanisms that are inconsistent with the experimental data.

Krupka and Laidler (5) have proposed a general reaction scheme for an inhibited system involving two enzyme-substrate intermediates. Eqs. 1–4 can be modified as

$$\begin{array}{ccccc} & & & & P_1 \\ & & & & + \\ E + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES & \xrightarrow{k_2} & ES' \xrightarrow{k_3} E + P_2 \\ + & & + & & + \\ \begin{matrix} I \\ \downarrow\uparrow \\ EI \end{matrix} & \xrightarrow{k_I} & \begin{matrix} I \\ \downarrow\uparrow \\ ESI \end{matrix} & \xrightarrow{K_I^S} & \begin{matrix} I \\ \downarrow\uparrow \\ ES'I \end{matrix} \xrightarrow{K_I'} \end{array} \qquad (7)$$

where ES is still the Michaelis-Menten addition complex and ES' is the acylenzyme (acetylenzyme for AChE). K_I is the dissociation constant of EI, K_I^S of ESI, and K_I' of $ES'I$. The equation for the reaction rate v in Lineweaver-Burk form is (26):

$$1/v = (K_M/V)(1 + [I]K_I)/[S] + (1 + [I]/K_I^*)/V \qquad (8)$$

where

$$1/K_I^* = 1/[K_I^S(1 + k_2/k_3)] + 1/[K_I'(1 + k_3/k_2)] \qquad (9)$$

In the traditional treatment, $1/K^*$ equals the first term on the right of Eq. 9 becomes the acylenzyme ES' and $ES'I$ are absent; that is, K' approaches infinity, k_2 in Eq. 7 is 0, k_3 in Eq. 7 simply becomes k_2 in Eq. 2, and $K_I^* = K_I^S$. If K_I^* also

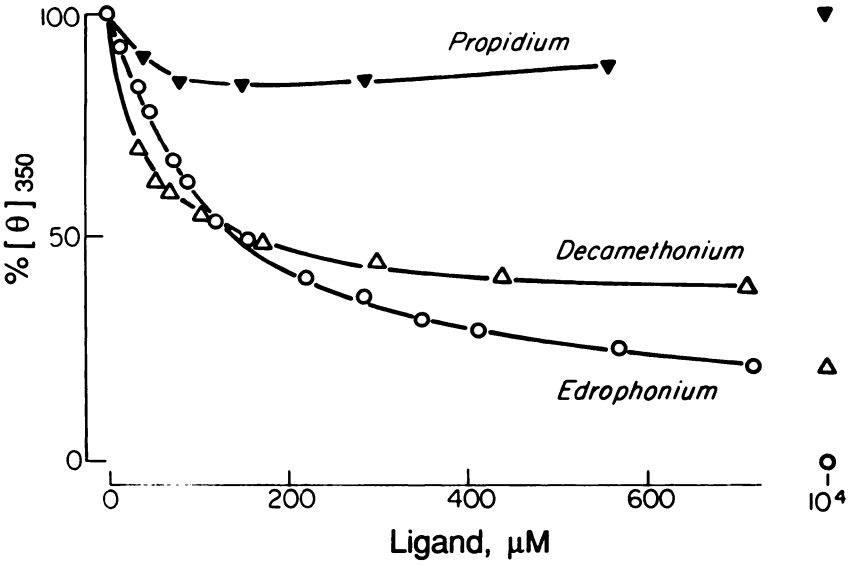

Fig. 9. Displacement of bound tacrine from AChE by competing ligands at 25°. AChE at 0.36 mg/ml (or 5.4 μM) was reacted with 11 μM tacrine for 10 min. Three milliliters of the mixture in a 1-cm cuvette were titrated with 1 to 10 mM competing ligands. The ellipticities were corrected for dilution. Data points at 10 mM ligands were obtained separately.

equals K_I, Eq. 8 in this case reduces to Eq. 6 for noncompetitive inhibition.

In the acylenzyme-based theory (5, 26), $1/K^*$ equals the second term on the right of Eq. 9 because the *ESI* complex does not exist; that is, K_I^S approaches infinity. Eqs. 8 and 9 combine to give

$$1/v = (K_M/V)(1 + [I]/K_I)/[S] + \{1 + [I]/K_I'(1 + k_3/k_2)\}/V \quad (10)$$

If $k_3 \ll k_2$ [e.g., $k_2/k_3 = 6$ (32)], and $K_I = K_I'$, Eq. 10 again reduces to Eq. 6 for noncompetitive inhibition. K_I and K_I^* (Eqs. 8 and 10) can be directly calculated from the slopes and intercepts in Fig. 1. Alternately, replotting the slopes and intercepts (on the ordinate) of the $1/v$ versus $1/[S]$ plots against $[I]$ leads to two new slopes, K_M/K_IV and $1/K^*V$, respectively (33). With K_M and V known from Fig. 1, both K_I and K_I^* can be determined. We found that for the inhibition of AChE by tacrine, $K_I = 8.5$ nM and $K_I^* = 9.5$ nM. Assuming $k_2/k_3 = 6$ (32) for both ACh and acetylthiocholine as the substrate, $K_I' = 8.2$ nM. The 10% difference between K_I and K_I^* could not be distinguished within experimental errors. Thus, these calculations again led to an apparent noncompetitive inhibition by AChE by tacrine. Suffice it to say, the acylenzyme-based theory is applicable to the AChE-tacrine system, but no single mechanism is unequivocal for kinetics treatment.

In the acylenzyme-based theory, the double reciprocal plots will approximately reach a common intercept on the ordinate, $(1/v)$ axis, if K^* in Eq. 8 is greater than about $10K_I$; that is, the inhibition becomes competitive (26). K_I and K_I^* in Eq. 8 are also equivalent to K (competitive) and K (uncompetitive) in Rosenberry and Bernhard's derivations (34).

Binding sites at the active center. Our results showed an apparent noncompetitive inhibition of AChE by tacrine, which qualitatively agreed with the finding by Patocka *et al.* (19) (however, our K_I was 8.5 nM as compared with the K_I of 2.2 μM reported by these authors). AChE has three binding sites at its active center. The esteratic site (site 1) is the locus for the formulation of the acetylenzyme. The anionic site (site 2) that binds the choline moiety of ACh is free in the acetylenzyme intermediate; this site can also bind many quaternary ammonium and cationic aromatic compounds (6, 29, 31, 35). A ligand that binds to site 2 and inhibits deacetylation will show noncompetitive inhibition because the inhibitor does not compete with the substrate. The hydrophobic site (site 2′) binds many hydrophobic substrates and inhibitors (36, 37). Charged quaternary ammonium compounds bound to site 2 generally exhibit competitive inhibition (6, 29, 31), whereas uncharged tertiary ammonium compounds usually show noncompetitive or mixed-type inhibition. This seeming contradiction has been attributed to the binding of these uncharged compounds to site 2 after the acylenzyme is formed (5, 26). However, there is no direct evidence for the binding of these compounds at site 2. Both the kinetic and structural differences between the charged and uncharged ammonium compounds seem to suggest that the binding of uncharged compounds to site 2′ rather than site 2 is quite plausible. The three binding sites at the active center of AChE may partially overlap one another. Thus, edrophonium and decamethonium can replace bound tacrine, which sterically hindered the approach of DFP at site 1. The unusually high affinity of tacrine toward AChE further suggests that the amino group of tacrine might also be involved in its interaction with another, not yet identified, site at the active center. The binding of tacrine to the peripheral site (site 3) distinct from the active center may be ruled out because high concentrations of propidium did not displace the bound tacrine on AChE (Fig. 9).

Tacrine bound to AChE induced an extrinsic CD band at 348 nm, which red-shifted the absorption bands of the free tacrine by 12 to 24 nm. This is supported by the results of difference absorption specra (Fig. 8). The binding did not affect the secondary structure of the enzyme but may alter the local conformation of the aromatic side chains of AChE, suggesting that there is an interaction between bound tacrine and Trp or Tyr of the enzyme. In their study of the binding of AChE with *N*-methylacridinium, Shinitzky *et al.* (38) reported a new absorption band at 470 nm, which they attributed to the formation of a charge-transfer complex between the ligand and a Trp residue in the active center of the enzyme. The binding of tacrine to AChE may involve a similar charge transfer effect,

although the binding sites for *N*-methylacridinium and tacrine may not be exactly the same.

Protective effect of tacrine on AChE and dynamics of bound tacrine. The binding of tacrine to AChE protected the enzyme from inactivation by DFP (Fig. 7). The smaller magnitude of the 348-nm band for the AChE-tacrine-DFP complex, compared with that for the AChE-tacrine complex (Fig. 4, *curves 2* and *3*), may be due to rearrangement of the tacrine ring to accommodate the binding of the DFP molecule at the esteratic site. This is in accord with the titration results of the AChE and AChE-DFP complex with tacrine, which showed the same stoichiometric binding. With DFP bound to AChE, tacrine may approach the binding site on the enzyme at a different angle than with free AChE, inasmuch as the increase in $[\Theta]_{348}$ with increasing tacrine concentration was smaller for the AChE-DFP complex than for the free enzyme. Further, the essentially identical difference absorption spectra of the AChE-tacrine complex and the AChE-tacrine-DFP complex (Fig. 8) indicated that no bound tacrine was displaced by the binding of DFP to the enzyme. The present results are consistent with the reorientation of the probe through occupation and internal motion of the active site.

The slow rate of change of $[\Theta]_{348}$ for the AChE-tacrine-DFP complex may be related to the rate of reaction of AChE with DFP, which was retarded by steric hindrance exerted by the bound tacrine. The reaction time was shortened with increasing concentration of DFP. The rate of change was more drastically decreased when DFP was substituted with Soman, a potent phosphorylating agent with a bimolecular reaction constant 6000 times greater than that of DFP (5.6×10^7 versus 9.5×10^3 Min^{-1}), according to Forsberg and Puu (39).

Tacrine has been used to treat intoxications with psychotropic drugs known to have strong anticholinergic effect (16) and, recently, to treat the Alzheimer's disease (17). These are based on the inhibitory effect of tacrine on the activity of AChE. The present study clearly showed that tacrine can protect AChE from inactivation by DFP. Whether it can equally protect AChE against the toxicity of nerve agents such as Soman remains to be investigated.

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