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SHORT COMMUNICATION

Propidium—a Fluorescence Probe for a Peripheral Anionic Site on Acetylcholinesterase

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SUMMARY

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Propidium (3,8-diamino-5,3′-diethylmethylamino-*n*-propyl-6-phenylphenanthridium) diiodide binds with high affinity to a purified acetylcholinesterase isolated by lytic procedures from *Torpedo californica*. Complex formation results in 10-fold enhancement of fluorescence for the ligand in the bound state. The fluorescent ligand can be dissociated from the enzyme by back-titration with *d*-tubocurarine and gallamine under conditions of low ionic strength ($\Gamma/2 \cong 0.001$). At higher ionic strength, in 0.1 M NaCl, 0.04 M $MgCl_2$, and 0.01 M Tris-Cl, pH 8.0 ($\Gamma/2 = 0.23$), the above ligands are relatively ineffective in dissociating propidium from the enzyme. Edrophonium, an inhibitor that appears specific for the active center, does not dissociate propidium under conditions of high and low ionic strength. This specificity is consistent with propidium acting as a fluorescence probe for a peripheral anionic site on acetylcholinesterase.

Steady-state kinetic studies have revealed that a wide variety of quaternary ammonium ligands interact with acetylcholinesterase at one of two available sites, designated the peripheral anionic site and the anionic subsite within the active center (1–4). Recent investigations using fluorescence spectroscopy have enabled one to examine directly the ligand binding specificity and stoichiometry at the two separate sites (5–8). However, to date fluorescence monitoring of ligand-acetylcholinesterase complex formation has been based upon either the greatly diminished ligand quantum yields associated with binding (5, 6) or ligand absorption spectra suitable to effect quenching of the tryptophanyl fluorescence in acetylcholinesterase (7, 8). Ligands which show an enhancement of fluorescence upon complex formation possess an inherent advantage, in that additional information can be obtained from the intrinsic fluorescence properties of the ligand in the bound state.

Phenanthridium derivatives are known to exhibit changes in fluorescence quantum yield when bound to macromolecules (9, 10). Within this group of compounds, ethidium bromide has been most extensively studied since, upon intercalating between nucleic acid base pairs, it gives rise to a large en-

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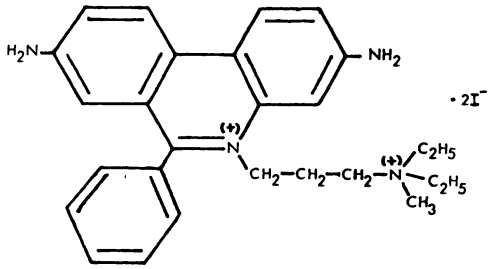

Propidium Diiodide

hancement of fluorescence. Propidium (3,8-diamino-5,3′-diethylmethylamino-*n*-propyl-6-phenylphenanthridium) diiodide, a congener of ethidium with an additional exocyclic quaternary nitrogen (I), was observed to be a potent inhibitor of acetylcholinesterase, exhibiting an affinity that is 1–2 orders of magnitude greater than ethidium. Thus propidium appeared to be an excellent candidate as a fluorescence probe of this enzyme.

Propidium diiodide was obtained from Calbiochem and used without further purification. Acetylcholinesterase from the electroplax of *Torpedo californica* was purified to apparent homogeneity as previously described (11). The enzyme has been designated as the lytic form, since its dissociation from electroplax membranes is brought about by mild tryptic treatment of the membrane fractions. Its molecular weight was estimated to be 330,000 and is composed of four similar, if not identical, subunits (11).

Fluorescence titrations were carried out in either 0.3-cm² cuvettes (0.2-ml total volume) or 1.0-cm² cuvettes (2-ml total volume) at 25° ± 0.2°. The sample chamber of the spectrofluorometer was modified with a four-cuvette rotating turret containing a reference in one position to correct for changes in excitation light intensity or inner filter effects. The excitation wavelength was 535 nm, and emission was measured at 602 nm. Further details on the fluorescence titration procedures for acetylcholinesterase have been presented previously (8). The enzyme activity was assayed by pH-stat methods. The buffer system used for the high ionic strength condition was 0.1 M NaCl 0.04 M $MgCl_2$, and 0.01 M Tris-Cl, pH 8.0, while 0.001 M Tris-Cl, pH 8.0, was employed for the low ionic strength titrations.

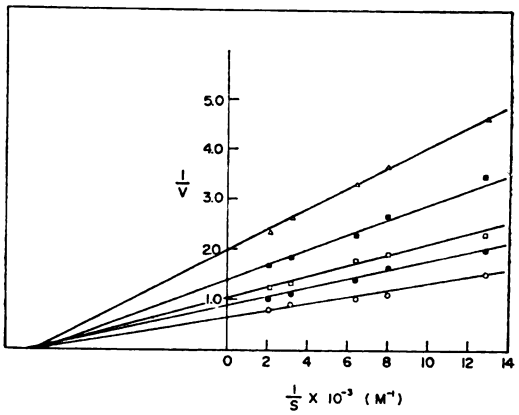

FIG. 1. *Lineweaver-Burk plot of propidium inhibition of acetylcholinesterase activity using acetylcholine as substrate*

Hydrolysis was measured by pH-stat methods at pH 8.0 in 0.1 M NaCl–0.04 M $MgCl_2$ at 25°. The purified *Torpedo* enzyme described in the text was used. O——O, no inhibitor; ●——●, 1.25 μM propidium; □——□, 2.5 μM propidium; ■——■, 5 μM propidium; △——△, 7.5 μM propidium. Slopes were determined from a least-squares analysis of the data.

Propidium inhibition of acetylcholinesterase-catalyzed acetylcholine hydrolysis exhibits noncompetitive kinetic behavior at high ionic strength (Fig. 1). Similar behavior also occurs at low ionic strength, but with a somewhat higher affinity for propidium (Table 1). While such kinetics is consistent with a peripheral site for propidium binding, these observations cannot establish that the propidium and substrate binding occur at nonoverlapping sites. As pointed out by Krupka and Laidler (12), preferential inhibitor binding to the active site of the acyl-enzyme intermediate can affect the deacylation rate and give the appearance of noncompetitive kinetics for the over-all reaction.

Figure 2a shows fluorescence titration profiles at low ionic strength for propidium in the absence and presence of various concentrations of purified acetylcholinesterase. The difference in limiting slopes reflects a 10-fold increase in the fluorescence of the ligand in the bound state. A fluorescence enhancement of this magnitude can be compared with the fluorescence changes (less than 2-fold) that we have observed for

TABLE 1

Fluorescence and steady-state kinetic measurements of dissociation constants for inhibitors of acetylcholinesterase

The low ionic strength media were 1.0 mM Tris-Cl, pH 8.0, for the fluorescence titrations and 1.0 mM NaCl for the kinetic studies. The high ionic strength medium was 100 mM NaCl, 40 mM $MgCl_2$, and 10 mM Tris-Cl, pH 8.0, for fluorescence studies. For the kinetic measurements no Tris-Cl was present.

Ligand	Buffer system	K_D (fluorescence)	K_I (inhibition kinetics)
		M	*M*
Propidium	Low ionic strength	2.9×10^{-7}	3.2×10^{-7}[a]
	High ionic strength	3.4×10^{-6}	3.8×10^{-6}[a]
Gallamine	Low ionic strength	3.8×10^{-7}	3.0×10^{-7}[b], 5.3×10^{-6}[c] 5.0×10^{-7}[d]
	High ionic strength	$>10^{-4}$	$>10^{-4}$[b],
d-Tubocurarine	Low ionic strength	4.6×10^{-6}	3.4×10^{-6}[c], 6.5×10^{-7}[d]

[a] Inhibition constants were calculated assuming noncompetitive behavior.

[b] Ref. 1, *Torpedo marmorata* acetylcholinesterase; low ionic strength $\Gamma/2 = 0.003$, high ionic strength 0.2 M $MgCl_2$.

[c] Ref. 2, *Electrophorus electricus* acetylcholinesterase; low ionic strength $\Gamma/2 = 0.009$.

[d] Ref. 3, Human erythrocyte acetylcholinesterase; low ionic strength $\Gamma/2 \leq 0.002$.

propidium in solvents of varying dielectric constants (ϵ = 6.1–78.5). Similar behavior has been noted for ethidium, where a 20-fold increase in fluorescence has been reported for the ligand when bound to DNA, yet smaller quantum yield changes were observed for the free ligand in a series of aliphatic alcohols and ethers (9). Thus fluorescence quantum yield changes of the magnitude seen for the associated propidium cannot be ascribed simply to a change in polarity on the enzyme surface but may be a consequence of preferential restriction in motion of one of the ring systems when bound to the macromolecule. A shift in excitation wavelength from 480 to 535 nm occurs upon complexation with acetylcholinesterase, while the emission maximum shifts only slightly, from 595 to 602 nm (uncorrected).

Binding of propidium appears stoichiometric with a 80,000–90,000 molecular weight species (Fig. 2a), which is in good agreement with our previous estimates of stoichiometry using bisquaternary inhibitors and serine-carbamoylating substrates (11). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate yields a subunit species whose molecular weight is 78,000–82,000 (11); thus it appears that a single propidium molecule binds to each of the four subunits on the enzyme. The dissociation constant calculated from fluorescence titrations is in reasonable accord with the K_I estimated from kinetic measurements (Table 1). Edrophonium [(3-hydroxyphenyl)dimethylethylammonium] chloride, a competitive inhibitor of acetylcholinesterase that appears specific for the active center, fails to cause appreciable dissociation of the enzyme-propidium complex (Fig. 2b). Both gallamine [1,2,3-tris(2-triethylammonium ethoxy)benzene triiodide] and *d*-tubocurarine appear to compete for the propidium binding site (Fig. 2c), and the affinity of these ligands for the enzyme may be calculated from the back-titration curves (13, 14). The dissociation constants so obtained are of the same magnitude as K_I values for *d*-tubocurarine or gallamine inhibition of acetylcholinesterases from various sources (Table 1).

At high ionic strength the affinity of the propidium-acetylcholinesterase complex is reduced (Fig. 3a); however, on the basis of the diminished competition by gallamine (Fig. 3c) and *d*-tubocurarine (not shown), the latter ligands would exhibit an even greater reduction in affinity for the propidium binding site at high ionic strength.

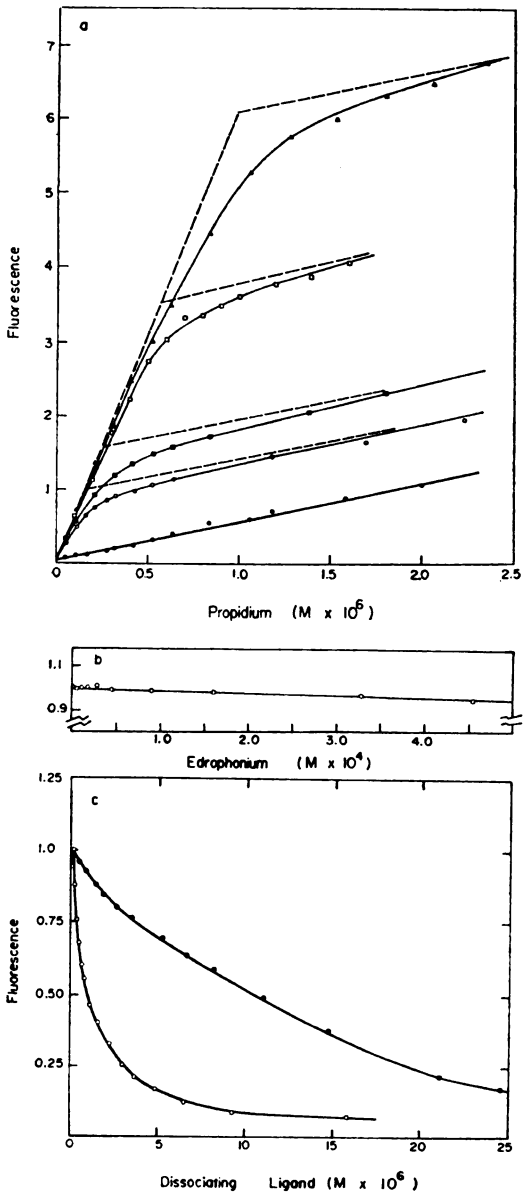

FIG. 2. *Fluorescence titrations of propidium diiodide-acetylcholinesterase complexes in 1 mM Tris-Cl, pH 8.0* ($\Gamma/2 \cong 0.001$)

a. Fluorescence enhancement of propidium diiodide in the presence of *Torpedo californica* acetylcholinesterase (lytic form). ●——●, blank titration in absence of enzyme; ○——○, 0.145 mg/ml of enzyme; ■——■, 0.24 mg/ml; □——□, 0.54 mg/ml; ▲——▲, 0.88 mg/ml.

b. Back-titration of the propidium-enzyme complex with edrophonium (8.0 μM propidium, 0.52 mg/ml of enzyme). c. Back-titration of the propidium-enzyme complex with gallamine (○——○) or *d*-tubocurarine (●——●) (7.2 μM propidium, 0.52 mg/ml of enzyme). All titrations were carried out at 25°. The excitation wavelength was 535 nm, and emission was measured at 602 nm. In the back-titrations the fluorescence of unbound propidium and the light scatter contribution of the enzyme were subtracted from the total fluorescence signal. Scatter contributions usually amounted to less than 8% of the total signal.

Edrophonium is also ineffective in dissociating propidium at high ionic strength (Fig. 3b).

A considerable body of evidence supports the contention that edrophonium binds specifically to the active center of acetylcholinesterase. Competitive inhibition is observed, with a K_I of 3.1×10^{-7} M for acetylcholine hydrolysis (15). Comparative rates of sulfonylation of the active center serine demonstrate that the bound edrophonium adopts an "endo" orientation consistent with steric occlusion of the esteratic serine (16). More recently, equilibrium dialysis measurements have shown a high-affinity binding site ($K_I = 2.5 \times 10^{-7}$ M) for edrophonium, with 1:1 stoichiometry between ligand binding sites and phosphorylatable active center serines (17). High-affinity binding of edrophonium is abolished by methanesulfonation of the active center esteratic site (17). In contrast, kinetic measurements with acyl and carbamoyl ester substrates suggest that gallamine and *d*-tubocurarine influence acetylcholinesterase activity by their association with an anionic site that is peripheral to the active center (1, 2). Moreover, these ligands do not form affine complexes with acetylcholinesterase at high ionic strength (1–3). Changeux has observed that gallamine at concentrations between 0.1 and 1 μM will cause partial inhibition of acetylcholinesterase, while in the presence of 0.2 M $MgCl_2$, 0.1–1 mM gallamine is required to produce equivalent effects on enzyme-catalyzed acetylcholine hydrolysis (1). Similar qualitative behavior is observed for the influence of gallamine on the carbamoylation and decarbamoylation rates of the active center serine (2).

Our findings that edrophonium does not dissociate propidium from acetylcholinester-

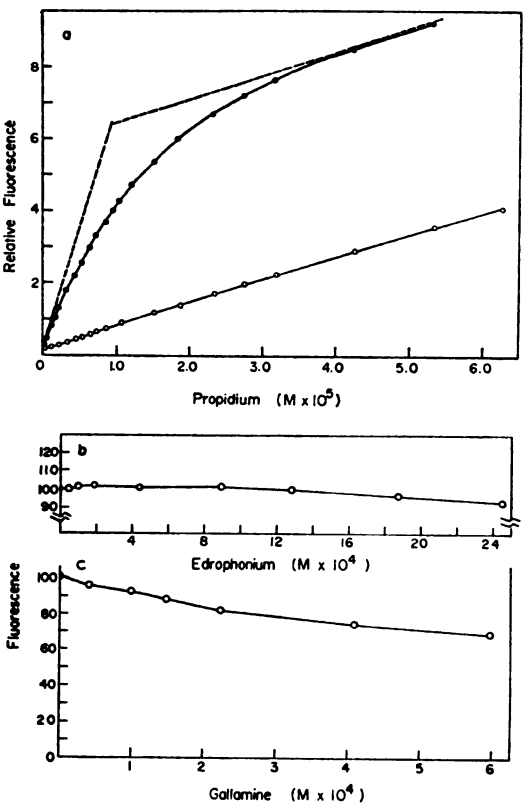

FIG. 3. *Fluorescence titration of propidium diiodide with acetylcholinesterase in 0.1 M NaCl, 0.04 M $MgCl_2$, and 10 mM Tris-Cl, pH 8.0* ($\Gamma/2 = 0.225$)
a. Fluorescence in the presence (O——O) and absence (●——●) of 0.76 mg/ml of enzyme. b. Back-titration of the propidium-enzyme complex with edrophonium (21.5 μM propidium, 0.88 mg/ml of enzyme). c. Back-titration with gallamine (O——O) (21.5 μM propidium, 0.88 mg/ml of enzyme). Other conditions were the same as those given in Fig. 2.

ase and that gallamine and *d*-tubocurarine only do so when the ionic strength is low would indicate that propidium preferentially associates with a peripheral anionic site on the enzyme. Competitive titration studies, when coupled with fluorescence lifetime measurements, should further delineate the binding specificity for various ligands on the multiple binding sites of acetylcholinesterase. Moreover, with the appropriate ligands, energy transfer measurements may enable one to calculate distances between the respective binding sites on the acetylcholinesterase molecule (8).

Finally, it is of some interest that the primary pharmacological manifestation *in vivo* of agents such as gallamine, *d*-tubocurarine, and pancuronium, which associate with a peripheral site on acetylcholinesterase (1, 2), is competitive neuromuscular blockade rather than acetylcholinesterase inhibition. Propidium administered subcutaneously to mice also causes flaccid paralysis. Using an inclined screen test, we found an ED_{50} for propidium in the Swiss-Webster strain of 4.3 mg/kg, which indicates that its potency is less than *d*-tubocurarine but comparable to gallamine. The avian response to neuromuscular blockade allows one to distinguish depolarizing from competitive blocking agents. The former elicit extension of the limbs and retraction of the neck, while the latter produce a flaccid paralysis (18). Propidium, when injected intravenously to chicks, produces flaccid paralysis, as do gallamine and *d*-tubocurarine, whereas with decamethonium the extensor response was observed. We could detect paralysis with 1.2 mg/kg of propidium. These initial observations indicate that certain phenanthridium derivatives may also serve as useful fluorescence probes for the isolated nicotinic receptor. A recent study on the interaction between dansylcholine and electroplax membrane fragments containing cholinergic receptors showed ligand dissociation constants between 15 and 45 μM, values much higher than those observed for most nicotinic agonists and antagonists (19). A fluorescence probe for the cholinergic receptor with a lower dissociation constant would also have a number of important applications.

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