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Spectroscopic Studies on Acetylcholinesterase: Influence of Peripheral-Site Occupation on Active-Center Conformation†

Harvey Alan Berman,* Wayne Becktel, and Palmer Taylor

ABSTRACT: Each subunit of acetylcholinesterase (AchE) contains, in addition to an active center, a peripheral anionic site at which a variety of structurally diverse cationic ligands associate. The influence of peripheral-site occupation on active-center conformation was assessed by examination of (pyrenebutyl methylphosphono)acetylcholinesterase (PBMP–AchE). In this conjugate, the pyrenebutyl moiety is linked covalently through a phosphonyl unit to the active-center serine. Association of propidium at the peripheral site results in quenching of ~88% of the pyrenebutyl fluorescence through dipolar excitation transfer; subsequent dissociation of propidium by competitive peripheral site ligands (e.g., gallamine, decamethonium, and *d*-tubocurarine) results in restoration of the pyrenebutyl fluorescence. Direct monitoring of propidium fluorescence provides an alternative demonstration of peripheral-site occupation and estimation of ligand affinity. The values of the dissociation constants calculated for gallamine and *d*-tubocurarine with PBMP–AchE are virtually equivalent with those determined for the native enzyme. In contrast, the association of decamethonium with PBMP–AchE is found to occur with a 50-fold lower affinity than with the native AchE; the decrease in affinity likely reflects steric occlusion of the active center by the bulky pyrenebutyl group. For the association of *d*-tubocurarine, although the competitive titration profile determined from the measurement of the propidium fluorescence is consistent with binding at a single high-affinity site, the titration profile determined through measurement of the pyrenebutyl fluorescence reveals an additional site of *d*-tubocurarine association. In the presence of low concentrations of *d*-tubocurarine, the fluorescence quantum yield of pyrenebutyl fluorescence is found to increase, whereas at higher ligand concentrations, pyrenebutyl fluorescence is quenched. Occupation of the high-affinity site which is competitive with propidium is required prior to occupation of the lower affinity site. The pyrenebutyl absorption spectrum of PBMP–AchE is shifted to longer wavelengths in the presence of gallamine and low concentrations of *d*-tubocurarine. Such spectral changes appear to be associated with binding exclusively at the peripheral anionic site. In the presence of decamethonium and high concentrations of *d*-tubocurarine, the pyrenebutyl spectrum undergoes striking alterations, and since under these conditions these ligands bind at the active center, the spectral perturbations likely reflect association of the cationic moieties within the vicinity of the active center. The intense optical activity exhibited in the circular dichroism spectra of the pyrenebutyl moiety of PBMP–AchE ($[\theta]_{348} = -4 \times 10^4$ deg cm^2/dmol) suggests that the chromophore is in apposition with a highly dissymmetric protein surface and that their relative orientations are dramatically altered upon occupation of either the peripheral site or the active center. These independent spectroscopic approaches reveal changes in active-site conformation that are associated with occupation of the spatially distinct peripheral anionic site and provide spectroscopic indexes which allow one to distinguish the site of ligand occupation.

Acetylcholinesterase (AchE)[1] exhibits a remarkable capacity to bind structurally diverse cationic ligands. In addition to the class of ligands which bind at the active center, there exists another class which associates at an anionic locus remote from the active center (Changeux, 1966). Although these ligands, which include the structures of gallamine, *d*-tubocurarine, and decamethonium, bind in a mutually exclusive manner at the peripheral anionic site, their modes of inhibition of catalysis are not equivalent and appear to be a property of the ligand as well as the site of occupation. Such behavior may suggest that either the occupation of the peripheral site by different ligands induces different active-center conformations or the peripheral site comprises a matrix of nonequivalent loci, all remote from the enzyme active center.

Spectroscopic probes have been employed to examine the microscopic environment and conformation of proteins (Stryer, 1968). When covalently situated at a distinct site, these probes

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[1] Abbreviations used: AchE, acetylcholinesterase; PBMPF, pyrenebutyl methylphosphonofluoridate; CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; dansyl, 8-(dimethylamino)-1-naphthalenesulfonate.

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become suitable for reporting on changes in the protein milieu that occur upon ligand association. A nucleophilic serine in the active center of AchE is particularly susceptible to phosphonylation and hence may be labeled by fluorescent phosphonates, as, for example, by pyrenebutyl methylphosphonofluoridate (PBMPF) which reacts to form the covalent conjugate PBMP–AchE (Berman & Taylor, 1978). This conjugate does not undergo appreciable "aging", exhibits a high fluorescence quantum yield, and, since the absorption spectrum of the conjugated pyrenebutyl moiety occurs at wavelengths (λ_{max} 348 nm) well removed from that of the protein absorption envelope, should serve as a suitable probe for analysis of active-center conformation through circular dichroism (Adler et al., 1973), ultraviolet (UV) difference spectroscopy (Donovan, 1973), and fluorescence spectroscopy.

The present paper examines the influence of peripheral-site occupation on the active-center conformation of AchE by employing fluorescence and absorption analysis of PBMP–AchE.

Materials and Methods

Materials. AchE from *Torpedo californica* was isolated as the 11S or "lytic" species by light trypsin digestion of electric organ membranes and purified to apparent homogeneity (Taylor et al., 1974). Preparation of the fluorescent conjugate PBMP–AchE has already been described (Berman & Taylor, 1978; Berman et al., 1980). After inhibition of the enzyme with PBMPF, the conjugate was passed through a Sephadex G-25 column to remove unreacted label and then dialyzed overnight against 2000 volumes of buffer. Stoichiometry of labeling was ascertained from the UV spectra of the conjugates employing the following extinction coefficients: pyrenebutyl, $\epsilon_{348} = 4 \times 10^4$ M^{-1} cm^{-1}; native AchE, $\epsilon^{280}_{1\%} = 17.5$ (Berman et al., 1980).

Spectroscopy. Corrected fluorescence spectra and equilibrium titrations were determined on a Farrand Mark I spectrofluorometer equipped with a corrected excitation source. Fluorescence titrations were carried out by using 1-cm^2 cuvettes positioned in a thermostated, multiturreted sample compartment. UV spectra were obtained on a Cary 16 spectrophotometer. UV difference spectra using tandem quartz cuvettes were obtained on a Cary 219 spectrophotometer.

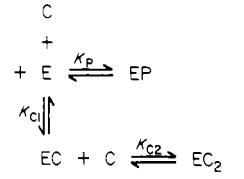
Circular dichroism data, obtained on a Cary 61 spectropolarimeter, are reported as the molar ellipticity $[\theta]_\lambda$ in degrees centimeter squared per decimole, which is calculated from the observed ellipticity, θ_{obsd} (degrees), and the molecular weight (MW) of the chromophore according to eq 1, where d and c

$$[\theta]_\lambda = \frac{\theta_{obsd} \times MW}{10dc} \quad (1)$$

denote, respectively, the path length in centimeters and chromophore concentration in grams per milliliter (Adler et al., 1973). For PBMP–AchE the molecular weight of the chromophore was chosen to be that of the pyrenebutyl moiety, 257. Molar ellipticities were calculated from the spectrum averaged over 15–40 acquisitions. The path length was 1 cm.

Competitive Titrations. All fluorescence values are corrected for dilution resulting from added titrant, lamp fluctuations, and any inner-filter effects and incident scatter. Solutions of ligands in buffer were prepared immediately before use and were added to solutions of AchE and PBMP–AchE containing propidium at concentrations 10–40 times its dissociation constant and at least 2 times the concentration of enzymes sites. The course of the competitive titrations was followed by monitoring the change in propidium fluorescence (λ_{ex} 535 nm; λ_{em} 625 nm) and from the influence of propidium dissociation on the fluorescence of the pyrenebutyl moiety (λ_{ex} 348 nm; λ_{em} 400 nm).

Scheme I



The dissociation constant for propidium diiodide and AchE in low ionic strength buffer (0.001 N Tris-HCl, pH 8) is found to be 1×10^{-7} M. Although an earlier published value is slightly higher, the equilibrium competitive relationships between peripheral-site ligands and propidium are essentially equivalent with those reported (Taylor & Lappi, 1975). The difference in propidium values probably results from assessing protein concentration by UV absorption and more exhaustive dialysis to assure low ionic strength and the absence of divalent cations prior to equilibrium titrations (cf. Materials and Methods). The presence of divalent cations has been shown to diminish the affinity of quaternary ammonium ligands for the peripheral site (cf. Taylor & Lappi, 1975).

Ligand Binding Data Analysis. Dissociation constants for the nonfluorescent ligands were derived from analysis of their capacities to displace the fluorescent ligand propidium for which the affinity is known (Berman & Taylor, 1978; Taylor & Lappi, 1975). Dissociation of propidium from PBMP–AchE diminishes the extent of energy transfer and hence results in a restoration of pyrenebutyl fluorescence. The observed fluorescence intensity of the pyrenebutyl chromophore, f, is related to the relative concentrations of free propidium, [P], and free competing ligand, [C], according to

$$\frac{f - f_P}{f_C - f} = \frac{K_P}{K_C}\frac{[C]}{[P]} \quad (2)$$

A logarithmic plot of $(f - f_P)/(f_C - f)$ vs. [C]/[P], which compares the fraction of the ligands bound with that of the fraction free and hence is analogous to the Hill equation, gives a slope of unity for a homogeneous class of independent sites. The value of K_C is obtained from the value at the x intercept and knowledge of K_P. The corresponding equation when the dissociation of propidium directly is monitored is obtained when the numerator and denominator of the left-hand term are multiplied by −1; in this case, f denotes propidium fluorescence.

For sequential association of the displacing ligand at two sites, one of which precludes propidium binding, Scheme I applies. At saturating concentrations of propidium, the fluorescence profile in the presence of C is described by

$$f = f_P + \frac{(f_1 - f_P)[EC]}{[EP] + [EC] + [EC_2]} + \frac{(f_2 - f_P)[EC_2]}{[EP] + [EC] + [EC_2]} \quad (3)$$

where [EP], [EC], and $[EC_2]$ denote the concentrations of enzyme complexes with propidium and with 1 and 2 equiv of competing ligand, respectively, and give rise to the corresponding fluorescence intensities f_p, f_1, and f_2. Equation 3 can be rewritten as

$$f = f_p + \frac{f_1 - f_p}{\frac{[P]}{K_P}\frac{K_{C1}}{[C]} + \frac{[C]}{K_{C2}} + 1} + \frac{f_2 - f_p}{\frac{[P]}{K_P}\frac{K_{C1}K_{C2}}{[C][C]} + \frac{K_{C2}}{[C]} + 1} \quad (4)$$

The corresponding equation for sequential association of C when propidium is absent is

$$f = f_0 + \frac{f_1 - f_0}{\frac{K_{C1}}{[C]} + \frac{[C]}{K_{C2}} + 1} + \frac{f_2 - f_0}{\frac{K_{C1}K_{C2}}{[C][C]} + \frac{K_{C2}}{[C]} + 1} \quad (5)$$

where f_0 is the pyrenebutyl fluorescence of PBMP-AchE in the absence of propidium.

In the competitive titrations, propidium was present at concentrations which were 10–40 times its dissociation constant. Titrations with competitive ligands to produce concentrations in excess of the enzyme and the dissociation constants allowed the free enzyme concentration to be neglected and the free ligand concentration to be approximated by the total ligand concentration added. When the concentrations of C or P are comparable with that of the enzyme, the quadratic equation must be used to calculate the concentration of free ligand.

Inspection of the second and third terms in eq 4 and 5 reveals the dependence of the competitive titration profile on the values of K_{C1} and K_{C2}, the relative changes in fluorescence upon complexation of one $(f_1 - f_p)$ and two $(f_2 - f_p)$ equivalents of ligand, and the concentration of propidium, when present. A plot of fluorescence vs. concentration of C for the second term would show a maximum whereas the quadratic dependence in the third term expresses a sigmoidal relationship between ligand binding and concentration. This relationship is reminiscent of the velocity–substrate concentration curves described for enzymes which bind multiple substrates in random order (Ferdinand, 1966).

The correspondence between the titration data and eq 4 and 5 was analyzed by Marquardt's nonlinear regression procedure (1963) where the initial, f_p, and final, f_2, values of fluorescence were derived from limiting values of the titration profiles. From the set of values of the observed fluorescence and the concentration of C, dissociation constants K_{C1} and K_{C2} and the maximum fluorescence of the first complex when all the higher affinity sites were saturated (f_1) were calculated until the change in each parameter was less than 1×10^{-8}.

Results

Equilibrium Competitive Titrations. Association of the nonfluorescent ligands gallamine, decamethonium, and *d*-tubocurarine with AchE and PBMP–AchE was measured from their capacity to displace the fluorescent ligand propidium. Dissociation of propidium was monitored *directly*, by noting changes in its fluorescence signal at 625 nm upon excitation at 535 nm, and *indirectly*, by noting the influence of propidium displacement on the intensity of the pyrenebutyl fluorescence at 400 nm upon excitation at 348 nm. The latter measurement is feasible due to overlap between the pyrenebutyl emission and propidium absorption spectra, which in turn permits resonance excitation transfer between these probes (cf. Berman et al., 1980). Association of propidium with PBMP–AchE results in quenching of ~88% of the initial pyrenebutyl fluorescence. Dissociation of propidium with saturating concentrations of a competitive ligand not possessing the appropriate spectral overlap is expected, in principle, to restore the pyrenebutyl fluorescence to values obtained prior to quenching by propidium provided that association of the competitive ligand does not influence the immediate environment in which the pyrenebutyl fluorophore resides.

Competitive dissociation by gallamine of the propidium complex with PBMP–AchE is illustrated in Figure 1. From examination of the titration profiles obtained by measuring changes in propidium fluorescence directly (panel A) or pyrenebutyl fluorescence (panel B), propidium dissociation by gallamine is seen to proceed to greater than 95% completion.

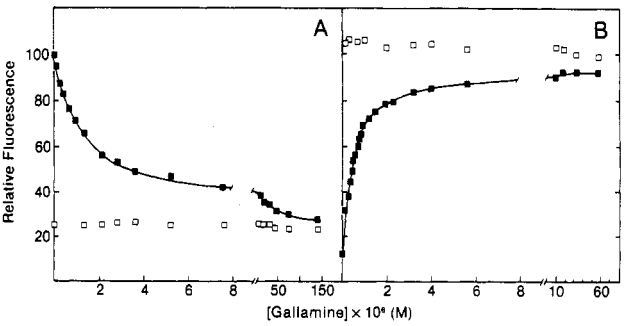


FIGURE 1: Propidium dissociation from (pyrenebutyl methylphosphono)acetylcholinesterase by gallamine. (A) Titration profile derived from measurement of propidium fluorescence (λ_{ex} 535 nm; λ_{em} 625 nm). (■) PBMP–AchE; (□) buffer. Propidium and enzyme were present at concentrations of 1.6×10^{-6} and 7.4×10^{-7} M, respectively. (B) Titration profile derived from measurement of pyrenebutyl fluorescence (λ_{ex} 348 nm; λ_{em} 400 nm). (■) PBMP–AchE in the presence of propidium; (□) PBMP–AchE in the absence of propidium. The initial enzyme concentration was 4.3×10^{-7} M, and the propidium concentration, when present, was 1.0×10^{-6} M. For the experiments described in both panels, the buffer was 0.001 N Tris-HCl, pH 8.

Association of gallamine with PBMP–AchE in the absence of propidium exerts no discernible effect on pyrenebutyl fluorescence up to 7×10^{-5} M ligand concentrations. Analyses of these data and those obtained for the ligands *d*-tubocurarine and decamethonium employing eq 2 are reported in Figure 2. The data show that the affinity of gallamine for PBMP–AchE derived from both methods of titration is equivalent and in close agreement with the value obtained from titration of the native enzyme.

Association of decamethonium with PBMP–AchE measured from analysis of the pyrenebutyl fluorescence profile is found to be equivalent with that determined from measurement of the propidium fluorescence; however, the dissociation constant calculated for decamethonium binding with PBMP–AchE is found to be 50-fold greater than that characteristic for association of this bisquaternary ligand with native AchE (Figure 2B). The marked reduction in affinity presumably reflects the influence of active-center modification by the bulky aromatic pyrenebutyl moiety.

Association of gallamine and decamethonium with PBMP–AchE exerted no discernible influence on the intrinsic fluorescence of the conjugated pyrenebutyl moiety. Also, the dissociation of propidium by these ligands is compatible with ligand binding at a single class of homogeneous sites.

Competitive titrations in the presence of *d*-tubocurarine where propidium dissociation was monitored directly gave nearly equivalent dissociation constants on native AchE and PBMP–AchE. Analysis of *d*-tubocurarine binding as measured from pyrenebutyl fluorescence (Figure 3), however, reveals that the fluorescence quantum yield of the pyrenebutyl moiety is enhanced in the presence of low concentrations of *d*-tubocurarine whereas it is partially quenched in the presence of higher concentrations of ligand. This biphasic behavior is apparent when ligand association is studied in the presence and absence of propidium. In the latter case, changes in pyrenebutyl fluorescence approaching 40% are observed. The biphasic titration profiles show characteristics expected of saturable, site-specific binding and hence are not consistent with the operation of dynamic collisional quenching mechanisms (Stern & Volmer, 1919).

Such complex behavior can be described by sequential ligand association at two distinct sites where occupation of the

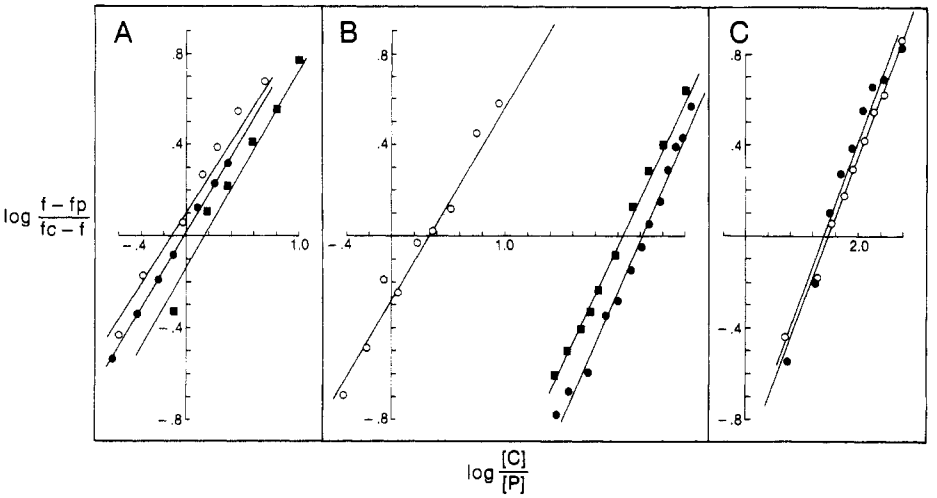


FIGURE 2: Logarithmic plots of $(f-f_p)/(f_c-f)$ vs. competitive ligand/propidium ratio for competitive dissociation of propidium from native and (pyrenebutyl methylphosphono)acetylcholinesterase by peripheral-site ligands. f denotes the pyrenebutyl fluorescence observed in the presence of both propidium and the competitive ligand, f_p the fluorescence with propidium only, and f_c the fluorescence in the presence of excess competitive ligand. Dissociation of propidium by gallamine (panel A), decamethonium (panel B), and *d*-tubocurarine (panel C) was measured in 0.001 N Tris-HCl buffer, pH 8. The square symbols refer to the measurement of pyrenebutyl fluorescence (λ_{ex} 348 nm; λ_{em} 400 nm). The circles refer to measurement of propidium fluorescence (λ_{ex} 535 nm; λ_{em} 625 nm); for this case, the ordinate axis is plotted as $(f_p-f)/(f-f_c)$ where f denotes propidium fluorescence (cf. Materials and Methods). Open symbols refer to measurements made on native AchE; filled symbols refer to measurements made on PBMP–AchE. Divisions on the x axis are marked every 0.2 unit.

high-affinity site occurs prior to occupation of the low-affinity site. Dissociation constants for the sequential equilibria described in eq 4 and 5 were obtained from nonlinear regression analysis according to Marquardt's formulation (1963) (see Materials and Methods). For the case where propidium is present, analysis of eq 4 reveals the presence of a high-affinity site for which the dissociation constant is calculated to be $\sim 5 \times 10^{-6}$ M, in good agreement with the value obtained from examination of the propidium fluorescence signal directly (Figure 2C), and a low-affinity site for which $K_{C2} = 4 \times 10^{-5}$ M.[2] For the case where propidium is absent, the enhancement and decrement in fluorescence occur in the same range of *d*-tubocurarine concentrations, but the smaller change in fluorescence makes it more difficult to calculate precise dissociation constants.

When the competitive titrations are carried out in buffers of high ionic strength, the biphasic character of *d*-tubocurarine association is abolished (Figure 3, inset). Dissociation of the propidium complex with PBMP–AchE in high ionic strength buffer (not shown) occurs in a manner compatible with competitive association at a single class of sites for which the dissociation constant is calculated to be 5×10^{-5} M and is consistent with the value obtained from study of *Electrophorus* AchE (Mooser & Sigman, 1974). Also, association of *d*-tubocurarine with the *Electrophorus* enzyme shows multisite attachment at low but not high ionic strength (Mooser & Sigman, 1974).

Ultraviolet Difference Spectra. UV difference spectra of PBMP–AchE in the presence of gallamine, decamethonium, and *d*-tubocurarine provide an independent assessment of the influence of peripheral-site ligands on the active-center environment. In the presence of gallamine, the pyrenebutyl absorption spectrum is shifted nearly 3 nm to longer wavelengths (Figure 4A,B). In the presence of decamethonium

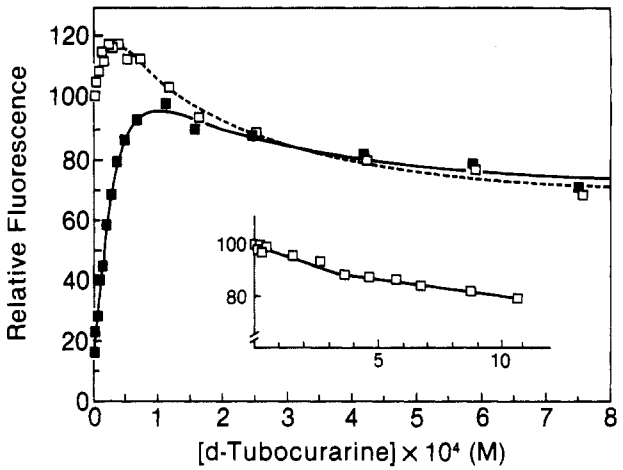


FIGURE 3: Association of *d*-tubocurarine with (pyrenebutyl methylphosphono)acetylcholinesterase in the presence and absence of propidium. (□) PBMP–AchE in the absence of propidium; (■) PBMP–AchE in the presence of 1.0×10^{-6} M propidium. The initial enzyme concentration in subunit sites was 5.0×10^{-7} M in 0.001 N Tris-HCl buffer, pH 8. The solid and dashed lines represent resolution of the data employing nonlinear regression analysis of ordered sequential ligand association. Resolution of the data obtained in the presence of propidium affords the following dissociation constants: $K_{C1} = 5.3 \times 10^{-6}$ M and $K_{C2} = 4.7 \times 10^{-5}$ M; the fluorescence value when only the high-affinity sites were occupied was derived to be 189 ± 25 relative to 12.8%. For the data obtained in the absence of propidium, nonlinear regression affords the following dissociation constants: $K_{C1} = 1.7 \times 10^{-5}$ M and $K_{C2} = 1.1 \times 10^{-4}$ M; the fluorescence value when only the high-affinity sites were occupied was derived to be 138 ± 21 relative to 100%. The inset shows the influence of *d*-tubocurarine association of the fluorescence of PBMP–AchE in Tris-HCl (0.010 N) buffer, pH 8, containing 0.1 N NaCl and 0.040 M $MgCl_2$. The initial enzyme concentration in subunit sites was 4.8×10^{-7} M. The axes are plotted to the same scale as in the parent figure.

(Figure 4C), the spectral perturbation is more complex and would be consistent with a spectral shift accompanied by either a hyperchromicity or a generalized spectral broadening. For both gallamine and decamethonium, the spectral changes exhibit saturable behavior and occur within a range of concentrations commensurate with the ligand dissociation constants (Table I).

[2] The data can also be described by a *parallel* ligand binding model in which E and C combine to form two distinct singly complexed species, EC_a and EC_b, which upon combination with another equivalent of C affords the doubly complexed species EC_2. Assigning dissociation constants and distinguishing this pathway from the *sequential* scheme are not feasible since the respective fluorescence quantum yields characteristic of EC_a and EC_b are not known.

Table I: Dissociation Constants Determined for Peripheral-Site Ligands with AchE and PBMP-AchE from Competitive Dissociation of Propidium[a]

ligand	AchE[b] K_c (M)	PBMP-AchE[b] K_c (M)	PBMP-AchE[c] K_c (M)
gallamine	$(0.7 \pm 0.3) \times 10^{-7}$	$(1.5 \pm 0.6) \times 10^{-7}$	$(1.0 \pm 0.8) \times 10^{-7}$
decamethonium	$(2.3 \pm 0.7) \times 10^{-7}$	$(1.1 \pm 0.5) \times 10^{-5}$	$(1.1 \pm 0.1) \times 10^{-5}$
d-tubocurarine	$(3.7 \pm 2.4) \times 10^{-6}$	$(6.3 \pm 1.7) \times 10^{-6}$	$(5.3 \pm 2.1) \times 10^{-6}$ [d] $(4.7 \pm 2.3) \times 10^{-5}$ [d]

[a] In all cases the enzyme was present in 0.001 N Tris-HCl buffer, pH 8. [b,c] Dissociation constants were calculated by using eq 2 and a value of $K_p = 1.0 \times 10^{-7}$ M for propidium. The values reported are the dissociation constants and standard deviations obtained from the average of at least five determinations. Association of peripheral site ligands with PBMP-AchE was followed by measuring changes in propidium fluorescence (b) or pyrenebutyl fluorescence (c). Logarithmic plots of eq 2 afforded straight lines for which the slopes were calculated to be in the range 0.9–1.1. [d] Dissociation constants were calculated by Marquardt analysis, assuming ordered sequential ligand association (see Materials and Methods).

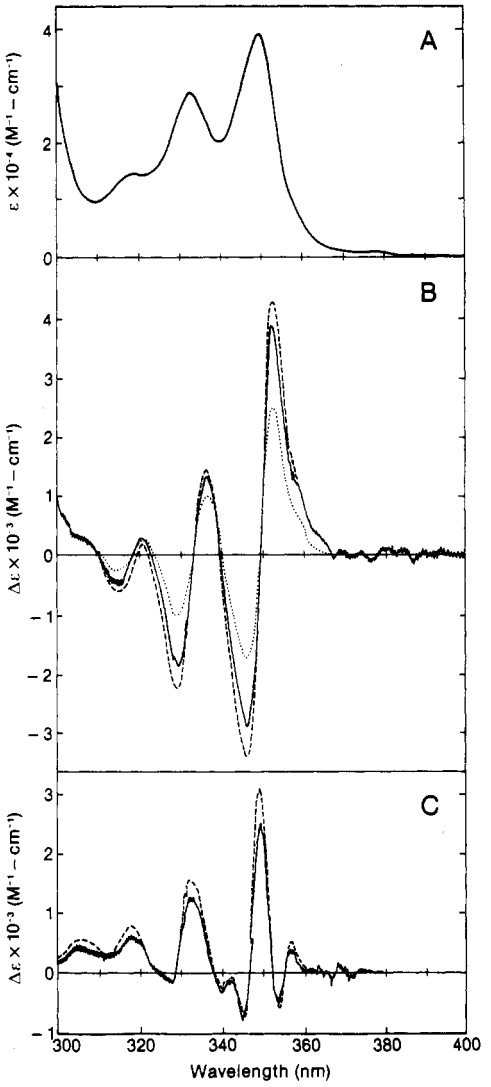


FIGURE 4: Ultraviolet difference spectra of (pyrenebutyl methylphosphono)acetylcholinesterase in the presence of gallamine and decamethonium. (A) The absorption spectrum of PBMP-AchE in the region between 300 and 400 nm. (B) UV difference spectra of PBMP-AchE in the presence of different concentrations of gallamine. The enzyme was present at a concentration of 8×10^{-6} M in subunit sites. Gallamine concentrations: (···) 3.2×10^{-6} M; (—) 6.4×10^{-6} M; (---) 2.08×10^{-5} M. (C) UV difference spectra of PBMP-AchE in the presence of different concentrations of decamethonium. The enzyme was present at a concentration of 8.1×10^{-6} M in subunit sites. For the respective curves, the concentrations of ligand are (—) 9.0×10^{-6} M and (---) 9.0×10^{-5} M. The buffer was 0.001 N Tris-HCl pH 8. The optical path length was 1 cm.

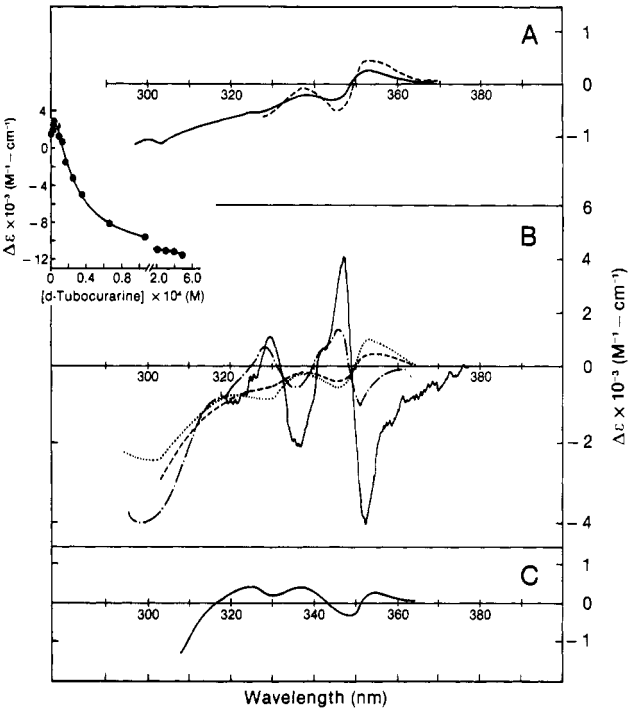


FIGURE 5: Ultraviolet difference spectra of (pyrenebutyl methylphosphono)acetylcholinesterase in the presence of *d*-tubocurarine. Panel A reports the differences obtained in the presence of concentrations of ligand which are equal to or less than stoichiometry. Panel B shows the spectra obtained when the ligand was present at concentrations greater than enzyme stoichiometry. The initial enzyme concentration was 4.9×10^{-6} M in subunit sites in 0.001 N Tris-HCl buffer, pH 8. *d*-Tubocurarine concentrations are the following: (A) (—) 2.06×10^{-6} M; (---) 5.10×10^{-6} M; (B) (···) 8.18×10^{-6} M; (---) 1.13×10^{-5} M; (·–) 2.56×10^{-5} M; (—) 2.11×10^{-4} M. The spectra were obtained by using tandem cuvettes where solutions of PBMP-AchE and buffer were placed in the front and rear compartments, respectively, of both the sample and reference cuvettes. Aliquots of ligand were added to the enzyme solution in the sample cuvette and to the rear compartment of the reference cuvette, while an equivalent volume of buffer was added to the enzyme compartment of the reference. The optical path length was 0.45 cm. (inset) Plot of the absolute difference in extinction coefficient at the longest wavelength vs. concentration of *d*-tubocurarine. The change in extinction is represented as positive when a bathochromic shift is observed and negative when a hypsochromic shift is observed. Panel C reports the UV difference spectra of PBMP-AchE in the presence of *d*-tubocurarine in a high ionic strength Tris-HCl (0.010 N) buffer, pH 8, containing 0.040 M $MgCl_2$ and 0.10 N NaCl. The enzyme was present at a concentration of 7.6×10^{-6} M. The *d*-tubocurarine concentration was 1.0×10^{-4} M. As in panels A and B, the optical path length was 0.45 cm.

In the presence of *d*-tubocurarine, the absorption spectrum of the pyrenebutyl moiety undergoes changes which depend on ligand concentration and ionic strength of the buffer. In low ionic strength buffer, low concentrations of *d*-tubocurarine (Figure 5A) cause a 1–2-nm red shift without any accompanying changes in the spectral intensity. With increasing concentrations of ligand (Figure 5B), however, the spectral difference gradually diminishes until, after reversal, the spectrum is shifted 1–2-nm to shorter wavelength. The

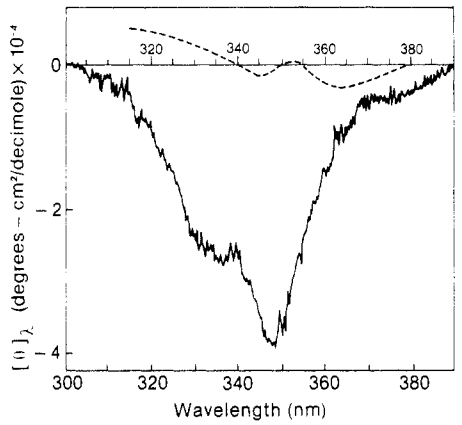


FIGURE 6: Circular dichroism spectra of (pyrenebutyl methylphosphono)acetylcholinesterase. The enzyme conjugate was present at a concentration of 1.6×10^{-5} M in subunits in 0.001 N Tris-HCl buffer, pH 8 (—), and in 8 M urea (- - -). The optical path length was 1 cm; the temperature was 20 °C.

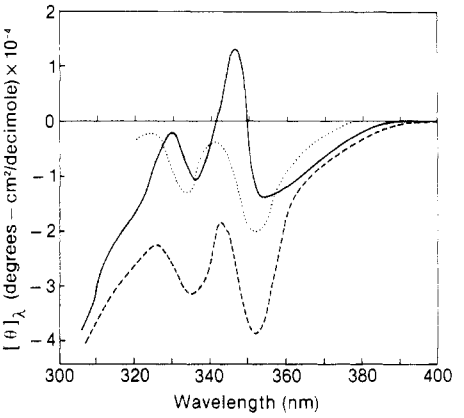


FIGURE 7: Circular dichroism spectra of (pyrenebutyl methylphosphono)acetylcholinesterase in the presence of peripheral-site ligands. Decamethonium (—), *d*-tubocurarine (···), and gallamine (- - -) were present at concentrations which were 20-, 2.5-, and 16-fold in excess of the enzyme concentration, which was typically in the range $(5–6) \times 10^{-6}$ M. The buffer was 0.001 N Tris-HCl, pH 8; the temperature was 20 °C. Each curve represents the spectrum obtained after averaging over 15 determinations.

maximal difference between the two states, therefore, is 4 nm. A plot of the total difference in extinction vs. concentration of ligand (inset) shows biphasic behavior similar to that observed in the fluorescence profile (Figure 3). In high ionic strength buffer (Figure 5C), such biphasic behavior is absent, and the presence of *d*-tubocurarine shifts the pyrenebutyl absorption spectrum to longer wavelengths, similar to the spectra observed in low ionic strength buffer at low concentrations of ligand (Figure 5A).

Circular Dichroism. Chiral relationships at the active center of PBMP–AchE were examined through measurement of the circular dichroism of the pyrenebutyl moiety. The circular dichroism spectrum of PBMP–AchE in the region of 300–400 nm shows the pyrenebutyl chromophore to exhibit intense optical activity, as represented by the molar ellipticity at 348 nm, $[\theta]_{348} = -4 \times 10^4$ deg cm^2/dmol (Figure 6). Upon denaturation of the conjugate in 8 M urea, the optical activity is lost, indicating that extrinsic rather than intrinsic Cotton effects prevail. Intrinsic effects arising from asymmetry at phosphorus six bond lengths removed from the pyrenebutyl chromophore are expected to be small.

The influence of peripheral-site ligands on the extrinsic dichroism of the conjugated pyrenebutyl chromophore is shown in Figure 7. In the presence of gallamine, the CD spectrum undergoes a bathochromic shift, as observed also in the UV difference spectra, and exhibits a negative minimum at 352 nm. The intensity of the spectrum is not substantially altered from that observed in the absence of ligand and indicates that gallamine binding at the peripheral site alters the active-center environment without significant effect on the extrinsic chirality at the active center. At concentrations of *d*-tubocurarine in 2.5-fold excess over the enzyme concentration (Figure 7), the CD spectrum appears at longer wavelengths and with a diminished intensity in comparison with that observed in the absence of ligand. At higher ligand concentrations, the spectra appear at shorter wavelengths and exhibit progressively diminished optical activity so that the final state achieved is blue shifted with reference to the state observed in the absence of ligand. Hence, the alterations induced in the CD spectra by *d*-tubocurarine show behavior and a concentration dependence similar to those observed in the UV absorption spectra. In the presence of decamethonium, the position and band shape of the CD spectrum are markedly altered. The spectrum exhibits, in addition to the characteristic bands of negative ellipticity, a maximum which is reversed in sign from the spectrum observed in the absence of exogenous ligand. Such a complex band shape may arise from multiple orientations of pyrenebutyl chromophores situated in environments of opposite chirality and where in one orientation the chromophore absorbs at shorter wavelengths than it does in the other. The observation of a diverse population of species in the presence of decamethonium and for which the spectra are blue shifted with reference to PBMP–AchE is consistent with the complex band shape observed in the UV difference spectrum (Figure 4C).

Discussion

Topographic Specificity of Peripheral-Site Ligands. AchE isolated by tryptic digestion from membranes of Torpedo electric organs is a tetrameric species composed of equivalent 80 000 molecular weight subunits. Stoichiometry of ligand association would suggest that each subunit contains an active center and a spatially distinct peripheral anionic site (Taylor & Lappi, 1975; Berman & Taylor, 1978). At the active center, some 4 Å removed from the catalytic serine is an anionic subsite (Nachmansohn & Wilson, 1951). In addition to substrates, cationic inhibitors such as edrophonium and *N*-methylacridinium bind at this site (Mooser & Sigman, 1974; Taylor & Lappi, 1975). Another class of ligands comprises the group of bisquaternary structures which associate at a locus denoted as the peripheral anionic site. Prototypic ligands in this class include *d*-tubocurarine, gallamine, and propidium. Energy-transfer measurements indicate that the covalent pyrenebutyl moiety at the active center and propidium are separated by a distance greater than 20 Å (Berman et al., 1980). Other bisquaternary ligands, e.g., decamethonium, where the cationic termini are separated by ~14 Å, can associate in a mutually exclusive manner with ligands both at the active-center and at the peripheral anionic site, yet their binding stoichiometries are one ligand per subunit. The distance between the sites determined spectroscopically is greater than the length of the extended decamethonium molecule, and two proposals have been put forward to account for competition of decamethonium at both of these sites (Berman et al., 1980).

Comparative features of ligand binding to AchE and PBMP–AchE demonstrate the topographic uniqueness of the peripheral and active-center sites. Association of gallamine and propidium with AchE is mutually exclusive at the peripheral site, and each ligand exhibits nearly equivalent affinities for native and active-center modified enzyme (Figure

2). Such behavior would be expected for ligands which associate solely at a site remote from the steric constraints imposed by modification of the active center. The affinity of decamethonium, in contrast, is 50-fold lower for PBMP–AchE than for AchE and indicates that modification of the active center occludes the anionic locus required for association of this ligand. Modification of the active-center serine with bulky groups has been observed to alter association of decamethonium and related bisquaternary ligands whereas modification with dimensionally compact alkyl phosphates and alkanesulfonates exerts only a negligible influence on ligand binding (Taylor & Jacobs, 1974).

Although the association of *d*-tubocurarine with AchE and PBMP–AchE appears equivalent when determined by measuring propidium displacement directly (Figure 2C), the complexity of ligand association is unmasked when pyrenebutyl fluorescence is monitored. In buffer of low ionic strength, the fluorescence titrations (Figure 3) and the profiles obtained from measurement of the UV difference spectra (Figure 5A,B) are biphasic and compatible with association of *d*-tubocurarine at more than one site per subunit. In buffer of higher ionic strength, the affinities of both sites are reduced, and in fact, it is possible to detect association at only one site. Despite the reduction in affinity, the difference spectrum generated from *d*-tubocurarine association at high ionic strength resembles that of the higher affinity of the two states seen at low ionic strength.

Influence of Peripheral-Site Occupation on Active-Center Conformation. Peripheral-site occupation of PBMP–AchE by gallamine, *d*-tubocurarine, and decamethonium markedly alters the absorption spectrum of the conjugated pyrenebutyl moiety. Examination of the comparative behavior of peripheral-site ligands reveals that those ligands which associate at the locus denoted as the peripheral anionic site, e.g., gallamine and (at low concentrations) *d*-tubocurarine, effect a shift in the pyrenebutyl spectrum to a longer wavelength, whereas those ligands, e.g., decamethonium and (at higher concentrations) *d*-tubocurarine, where there is independent evidence for association at the active center effect a shift to shorter wavelengths. Hence, association of peripheral-site-specific ligands produces spectra distinct from those elicited by active-site ligands. A similar conclusion was derived from study of the AchE conjugate with (dansylamido)pentyl methylphosphonofluoridate for which slow, but reversible, changes induced by transition metals in fluorescence of the dansyl probe conjugated at the active center (Epstein et al., 1979) could be correlated with corresponding inhibition of catalytic activity (Pattison & Bernhard, 1978).

Production of characteristically different spectra for the conjugated pyrenebutyl moiety from ligand occupation of the peripheral site or two sites on AchE is not surprising since the individual ligands have disparate modes of inhibition of catalysis. Propidium and gallamine, which by titration criteria are found to bind only to the peripheral site, show uncompetitive components in their inhibition of both steady-state catalysis and enzyme carbamoylation by cationic substrates (Changeux, 1966; Crone, 1973; Roufagalis & Quist, 1972; Taylor & Lappi, 1975). Competitive fluorescence titration measurements indicate that *d*-tubocurarine can occupy two sites simultaneously (Mooser & Sigman, 1974) but with the higher affinity site being competitive with peripheral-site ligands (Taylor & Lappi, 1975). *d*-Tubocurarine inhibition of AchE catalysis and carbamoylation by cationic substrates reflects its binding to more than a single site, one of which is competitive with substrates (Mooser & Sigman, 1974). The flexible bisquaternary ligands of the decamethonium prototype are competitive inhibitors of the enzyme for both quaternary ester and carbamoylating substrates (Mooser & Sigman, 1974; Rosenberry & Bernhard, 1972; Changeux, 1966). In addition to these kinetic criteria, thermodynamic data show that one of the cationic groups of the bisquaternary ligands binds at the anionic subsite in the active center (Taylor & Jacobs, 1974; Mooser & Sigman, 1974). Despite the rather different modes of inhibition of the enzyme, decamethonium and gallamine antagonize each other's binding to the enzyme as measured by gallamine's reversal of bisquaternary inhibition of AchE (Changeux, 1966) and by competitive titrations with propidium (Taylor & Lappi, 1975). Analysis of the complexities of the enzyme inhibition kinetics and the ionic strength dependence of binding for the various ligands indicates that the peripheral site may consist of a matrix of partially overlapping loci and occupation of the peripheral site may induce different conformations in the active center (Rosenberry, 1975a,b; Taylor & Lappi, 1975). Even in the simplest case, where the ligand associates only at a single site peripheral to the active center, the induced conformational changes and inhibitory kinetics will differ among the various ligands.

The spectroscopic studies reported here clearly establish that occupation of the spatially remote peripheral site can affect the configuration of acyl substrates covalently linked to the active-site serine. Moveover, a comparison of the spectral changes induced in the conjugated pyrenebutyl moiety detected by fluorescence emission, ultraviolet absorption, and circular dichroism enable one not only to distinguish the site of occupation of the perturbing ligand but also to describe, in part, the nature of the change in active-site configuration.

First, the intense molar ellipticity characteristic of the conjugated pyrenebutyl moiety indicates that it is tightly associated with a dissymmetric protein surface rather than being free to interact with the aqueous medium. Circularly polarized luminescence studies indicate that a pyrenebutyl phosphoryl conjugate with AchE from electric eel exhibits a high emission anisotropy factor, consistent with the CD findings reported here (Amitai et al., 1980). A defined position or multiple positions can be expected to arise from hydrophobic interactions between the pyrenebutyl moiety and aromatic amino acid side chains within the active center. A buried and rigidly held pyrenebutyl moiety is also consistent with the low frequency of collisional quenching of PBMP–AchE fluorescence when quenching of the covalent and free pyrenebutyl moiety by small anions, cations, and neutral species is compared (Berman & Taylor, 1978). Changes in fluoresence intensity of the long-lived pyrenebutyl moiety arising from peripheral-site occupation are difficult to interpret in molecular terms since the fluorophore is susceptible to quenching through diverse mechanisms including collisional deactivation and alteration in exposure to solvent. On the other hand, the absorption maxima of the pyrenebutyl chromophore correlate closely with the polarizability of the solvent (Berman & Taylor, 1978), and the position of the long-wavelength maxima observed for PBMP–AchE (λ_{max} 348 nm, compared with λ_{max} 341 nm in H_2O) corresponds with the chromophore being situated in an environment where hydrophobic forces and association with aromatic substituents prevail. In this connection, Shinitzky and co-workers (1973) have acquired evidence for aromatic cations reversibly bound at the active center serving as charge-transfer acceptors for donor tryptophanyl (indole) residues in *Electrophorus* AchE.

Difference spectra and changes in molar ellipticity of the pyrenebutyl chromophore that result from association of lig-

ands such as gallamine and (at low concentrations) *d*-tubocurarine at the peripheral anionic site exhibit a simple bathochromic shift without major additional perturbations in the spectra. Thus, the peripheral-site ligands would appear to induce a further increase in the polarizability of the pyrenebutyl environment. Such differences could arise simply from torsional movements of aromatic side chains in the vicinity of the pyrenebutyl moiety.

In contrast, association of decamethonium and (at high concentrations) *d*-tubocurarine, ligands for which there is evidence for their binding at the active center, produces more complex perturbations in the ultraviolet spectrum and the ellipticity (Figures 5 and 7). While a small hypsochromic shift is evident, the large perturbations in the spectra suggest a change in the orientations of the covalent chromophore. The tetrahedrally disposed alkyl phosphonates such as PBMPF can be considered as transition-state analogues for the rate-limiting step of acylation in the action of serine proteases (cf. Kraut, 1977), and in PBMP–AchE the conjugated pyrenebutyl group would be directed toward the anionic subsite rather than the spatially constrained acyl pocket within the active center (Taylor & Jacobs, 1974; Berman & Taylor, 1978; Berman et al., 1980). The large reduction in binding free energy seen for decamethonium association with the pyrenebutyl conjugated enzyme likely reflects nonoptimal positioning of one cationic head group of the decamethonium molecule in the vicinity of the active center as a consequence of direct steric interference by the conjugated pyrenebutyl moiety. It is also known that certain planar aromatic cations such as *N*-methylacridinium and 2-pyridinium aldoxime methiodide inhibit AchE activity toward acetylcholine and other quaternary ammonium esters but actually enhance AchE activity toward certain short-chain neutral esters such as methyl and ethyl acetate (Barnett & Rosenberry, 1977). Kinetic as well as thermodynamic titration data indicate that these cationic ligands associate at the active center. Hence, the region of the active center simultaneously accommodating the neutral ester moiety and the cationic effector may exhibit conformational flexibility. This propensity for adaptability in the fitting of ligands at the active center is consistent with the rate-limiting induced-fit mechanism proposed to account for hydrolysis of neutral substrates (Rosenberry, 1975b) and may also be responsible for the inhibitory synergism seen with neutral aromatic and cationic inhibitors (Rosenberry & Bernhard, 1972). Considerable evidence has been adduced for the binding of decamethonium and (at high concentrations) *d*-tubocurarine at the anionic site within the active center, and the close apposition of the cationic substituent on these ligands and the conjugated pyrenebutyl moiety provides an explanation for the striking spectral changes seen in the pyrenebutyl difference spectra and the circular dichroism data.

We have demonstrated previously that energy transfer from the pyrenebutyl moiety at the active center to propidium located at the peripheral anionic site could be employed to estimate the intersite distance separating these ligands (Berman et al., 1980). Since the energy-transfer efficiency measured from donor quenching exceeded that estimated from acceptor sensitization, a portion of the donor quenching was occurring through mechanisms other than dipolar excitation transfer (Förster, 1965). The additional donor quenching was attributed to the influence of peripheral-site occupation on the configuration and, in turn, the fluorescence quantum yield of the pyrenebutyl moiety at the active center. The observations presented here support this contention since other peripheral-site ligands which, unlike propidium, do not possess requisite spectral overlap with pyrenebutyl emission for dipolar energy transfer alter the quantum yield of the conjugated pyrenebutyl fluorophore. Concomitant changes in CD and UV difference spectra correlate with the fluorescence changes and serve to document the allosteric influence of peripheral-site occupation on active-center conformation.

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