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Role of the Peripheral Anionic Site on Acetylcholinesterase: Inhibition by Substrates and Coumarin Derivatives

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SUMMARY

Propidium has been demonstrated in previous studies to be a selective ligand for the peripheral anionic site on acetylcholinesterase (EC 3.1.1.7). Its association with this site can be advantageously monitored by direct fluorescent titration. We have measured the ability of acetylcholine, acetylthiocholine, haloxon [di-(2-chloroethyl)3-chloro-4-methylcoumarin-7-ylphosphate], and a coumarin derivative (3-chloro-7-hydroxy-4-methylcoumarin) to dissociate propidium from the peripheral anionic site of *Torpedo californica* acetylcholinesterase. Measurements were made by back-titration of propidium after complete inhibition of the active center with diisopropylfluorophosphate. Both acetylcholine and acetylthiocholine show substrate inhibition at high substrate concentrations. The concentrations required for occupation of the peripheral site, as ascertained by competition with propidium, correlated well with the concentration dependence for the kinetics of substrate inhibition. These observations are consistent with substrate inhibition being due to binding of acetylcholine or acetylthiocholine at a peripheral anionic site. Displacement of propidium by haloxon and coumarin indicated that these inhibitors also bind to the peripheral anionic site. The dissociation constants ascertained from peripheral site occupation are in agreement with the constants obtained from inhibition kinetics. Evidence is presented that competition with propidium obtained by direct fluorescence titrations, when combined with inhibition kinetics, provides a more reliable means for ascertaining site selectivity of various inhibitors than does a kinetic analysis alone.

Substantial evidence exists that AChE can be allosterically regulated by ligand binding to a site physically removed from the active center (1–5). Early studies of substrate catalysis showed that high concentrations of ACh were inhibitory to AChE (6–8) and this behavior, along with substrate specificity, distinguishes AChE from butyrylcholinesterase (EC 3.1.1.8) (7, 8). It has been proposed that substrate inhibition occurs when a second molecule of substrate binds to the anionic subsite of the active center. Binding was assumed to occur in either the Michaelis complex (7) or the acylenzyme (9–11), thus either competing with another substrate molecule for the correct orientation or slowing down the deacylation step.

An alternative proposal is that the substrate, at high concentrations, binds to the peripheral site and, thus, alters the active center conformation in a manner to decrease substrate binding or catalytic constants (1, 5, 12).

A variety of inhibitors interact with AChE. Included among them are a series whose inhibitory parameters could not be explained by simple competitive or noncompetitive inhibition (4, 5, 13). Rather, these ligands appear to act at a site peripheral to the active center. Definitive evidence for these considerations has come from the development of fluorescent ligands (3, 14), whose binding to the enzyme can be detected directly. In particular, propidium was shown to inhibit catalysis in a manner consistent with association at an allosteric site (3). Moreover, direct fluorescence titrations show that its binding is not affected when either reversible or irreversible ligands are bound at the active center (3, 15, 16). Finally, fluorescence energy transfer measurements reveal that the peripheral site is some 20 Å removed from the active center (17). Allosteric regulation of the active center conformation can also be shown by formation of a fluorescent phosphonyl enzyme by attachment of a fluorescent alkylphosphonate to the active site serine (18–20). Under these circumstances, peripheral site ligands, which in themselves neither are fluorescent nor exhibit spectroscopic properties suitable for energy transfer with the active center ligands, will alter the fluorescence emission of the ligand conjugated at the active center. This behavior reflects peripheral

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ABBREVIATIONS: AChE, acetylcholinesterase; ACh, acetylcholine; ATCh, acetylthiocholine; coumarin, 3-chloro-7-hydroxy-4-methylcoumarin; DFP, diisopropylfluorophosphate; DFP-AChE, acetylcholinesterase phosphorylated by diisopropylfluorophosphate; edrophonium, 3-hydroxy-*N*-ethyl dimethyl anilinium; haloxon, di-(2-chloroethyl)3-chloro-4-methylcoumarin-7-yl phosphate; M7C, *N*-methyl-7-carbamoxy-quinolinium; TMA, tetramethylammonium.

site alteration of active center conformation and is mediated allosterically through the enzyme subunit.

To examine the relationship between substrate inhibition and peripheral site occupation, binding of substrates and other ligands was studied by direct fluorescence titration and compared with catalytic parameters obtained by substrate hydrolysis. The findings presented here indicate that substrate inhibition can be explained by binding of substrates to the peripheral anionic site identified with propidium. In addition, we have examined the influence of propidium on haloxon and coumarin binding. Previous kinetic studies have shown that haloxon and coumarin compete with ACh and ATCh at the substrate inhibition site, which was postulated to be an allosteric site. Binding of haloxon to that site affected the ability of haloxon to phosphorylate the active center (5, 12, 21). Our findings also indicate that haloxon and coumarin show mutually exclusive binding with propidium, establishing that they bind to a peripheral site on AChE.

Experimental Procedures

Materials. The 11 S form of AChE was purified from *Torpedo californica* electric organs, as described earlier (22), and stored frozen in small aliquots. For the fluorescence titrations involving propidium, the enzyme was treated further with micrococcal nuclease and rerun on the affinity columns to eliminate traces of nucleic acids, which interfere with the fluorescence titrations (3). DFP-AChE was prepared by reaction with 1–2 mM DFP until inhibition was complete; the phosphorylated enzyme was then allowed to age at 4° for at least 24 hr. Aging results in loss of one of the isopropoxy groups. Excess DFP and the reaction products were removed by dialysis, and the modified enzyme was frozen in small aliquots. Completion of aging was confirmed by the inability of 2-(hydroxyimino)methyl-1-methylpyridinium to reactivate the enzyme.

Propidium iodide (Calbiochem) was dissolved in 1.0 mM Tris buffer, pH 8.0, and haloxon and coumarin (Cooper Technical Bureau, Berkhamsted, England) in ethanol, all as 3.0 mM stock solutions. Haloxon and coumarin were purified by repeated recrystallization (12). ATCh, ACh, TMA, and edrophonium stock solutions were prepared in water and used immediately after preparation.

Assays of enzyme activity. The method of Ellman *et al.* (23) was employed to measure ATCh hydrolysis. The reaction was started by addition of the substrate to the medium, containing enzyme in 0.1% bovine serum albumin and buffer. When reversible inhibition by haloxon was measured, the enzyme was added last, to prevent its phosphorylation in the absence of the substrate.

The pH-stat titrimetric assay was used to measure ACh hydrolysis at pH 8.0. The assay medium was 0.1 M NaCl, containing enzyme in 0.1% bovine serum albumin. Measured activities were corrected for nonenzymic substrate hydrolysis. All measurements were done at room temperature.

Fluorescence titrations. These experiments were performed on a Spex Fluorolog 2 spectrofluorometer interfaced to a Spex DM1B computer. Titrations were performed in 1-cm² cuvettes containing a magnetic stir bar. Propidium fluorescence was measured using excitation at 535 nm and emission at 640 nm. Measured fluorescence was corrected for changes in excitation energy, inner filter effects, and changes in sample volumes during titration by parallel titrations of appropriate sample blanks. For competitive displacement studies, propidium was present in 10–50 times excess over its dissociation constant and in excess over the concentration of the enzyme sites. Under these constraints, fluorescence data were processed using the following equation (3):

$$(f_p - f)/(f - f_c) = K_p/K_c\,[C]/[P] \qquad (1)$$

where f, f_p, and f_c denote fluorescence intensities during titration, with sites saturated with propidium, and with sites saturated with competing ligand, respectively; K_p and K_c are dissociation constants for propidium (P) and competing ligand (C).

Kinetic equations. Kinetic equations were analyzed to ascertain whether the experimental results conform to one of the two reaction schemes presented below. The first scheme describes competition between substrate and reversible inhibitor for the active center, whereas the second describes competition between substrate and reversible inhibitor for the peripheral site. Both schemes assume that substrate additionally binds to the enzyme peripheral site, thus inhibiting its hydrolysis at the active center.

When the inhibitor (I) binds only to the enzyme active center (EI), and the substrate (S) binds to the active center (ES) and peripheral site (SE), the following scheme may be constructed:

Scheme I

$$\begin{array}{ccccc} EI & \overset{K_{AI}}{\rightleftharpoons} & I + E & \overset{K_{AS}}{\rightleftharpoons} & ES \overset{k}{\rightarrow} E + P \\ + & & + & & + \\ S & & S & & S \\ \Vert\, K''_{PS} & & \Vert\, K_{PS} & & \Vert\, K'_{PS} \\ SEI & \overset{K'_{AI}}{\rightleftharpoons} & I + SE + S & \overset{K'_{AS}}{\rightleftharpoons} & SES \overset{k'}{\rightarrow} SE + P \end{array}$$

K_{AI} and K'_{AI} are the dissociation constants for the inhibitor binding to the active center, K_{PS}, K'_{PS}, and K''_{PS} are the dissociation constants for the substrate binding to the peripheral site, and K_{AS} and K'_{AS} are the dissociation constants for the substrate binding to the active center. The acylated enzyme was not included in the reaction scheme, because the kinetic experiments do not necessarily distinguish between binding to the nonacylated and acylated enzyme.

If the following assumptions[1] are introduced into Scheme I:

$$K_{PS} = K'_{PS} = K''_{PS}$$

$$K_{AS} = K'_{AS}$$

$$K_{AI} = K'_{AI}$$

$$k' = 0$$

it can be described by the simple equation:

$$\frac{v \cdot [I]}{v_0 - v} = K_{AI}\left(1 + \frac{[S]}{K_{AS}}\right) \qquad (2)$$

This equation was previously derived by Hunter and Downs (24) for the description of pure competitive reversible inhibition. Its left side is a linear function of $[S]$. When the substrate is ATCh or ACh, the rate constant k' is known to be much smaller that k (see Refs. 5 and 12) and the assumption that k' approaches zero, made in the above equation, thus seems justified.

If the inhibitor in Scheme I does not bind to E and SE with the same affinity, i.e., if K_{AI} is not equal to K'_{AI}, but the other assumptions introduced above still hold, Scheme I can be described by the following equation:

$$\frac{v \cdot [I]}{v_0 - v} = K_{AI}\left(1 + \frac{[S]}{K_{AS}}\right)\frac{\left(1 + \frac{[S]}{K_{PS}}\right)}{\left(1 + \frac{[S] \cdot K_{AI}}{K_{PS} \cdot K'_{AI}}\right)} \qquad (3)$$

Eq. 3 reduces to Eq. 2 when $K_{AI} = K'_{AI}$, but when $K_{AI} > K'_{AI}$ the left side of the equation is a nonlinear function of $[S]$, curved downwards with increasing $[S]$.

When the inhibitor (I) binds only to the enzyme peripheral site

[1] As pointed out by a reviewer, another way to partially simplify the equations derived from Scheme I is to apply microscopic reversibility constraint (applicable to cyclic schemes), instead of assumption of $K_{PS} = K'_{PS} = K''_{PS}$.

(IE), and the substrate (S) binds to the active center (ES) and peripheral site (SE), the following scheme may be constructed:

Scheme II

$$\begin{array}{ccccc} IE & + S \overset{K'_{AS}}{\rightleftharpoons} & IES & \overset{k''}{\rightarrow} & IE + P \\ K_{PI} \downarrow\uparrow & & \downarrow\uparrow K'_{PI} & & \\ I & & I & & \\ + & & + & & \\ E + S & \overset{K_{AS}}{\rightleftharpoons} & ES & \overset{k}{\rightarrow} & E + P \\ + & & + & & \\ S & & S & & \\ K_{PS} \downarrow\uparrow & & \downarrow\uparrow K'_{PS} & & \\ SE + S & \overset{K'_{AS}}{\rightleftharpoons} & SES & \overset{k'}{\rightarrow} & SE + P \end{array}$$

K_{PI} and K'_{PI} are the dissociation constants for the inhibitor binding to the peripheral site, and K_{AS}, K'_{AS}, and K''_{AS} are the dissociation constants for the substrate binding to the active center. The above scheme is described by the following equation:

$$\frac{v\cdot[I]}{v_0 - v} = \frac{\frac{K_{PI}\cdot[S]}{K_{AS}}\left(k + \frac{k'\cdot[S]}{K'_{PS}} + \frac{k''\cdot[I]}{K'_{PI}}\right)\left(1 + \frac{K_{AS}}{[S]} + \frac{[S]\cdot K_{AS}}{K'_{AS}\cdot K_{PS}} + \frac{K'_{AS}}{K'_{PS}}\right)}{\left(k + \frac{k'\cdot[S]}{K'_{PS}}\right)\left(\frac{K''_{AS}\cdot K_{PI}}{K'_{PI}\cdot K_{AS}} + \frac{[S]}{K''_{AS}}\right) - \frac{k''}{K'_{PI}}\left(1 + \frac{K_{AS}}{[S]} + \frac{[S]\cdot K_{AS}}{K'_{AS}\cdot K_{PS}} + \frac{K'_{AS}}{K'_{PS}}\right)} \quad (4)$$

This equation cannot be experimentally verified by measurement of enzyme activities, in the absence (v_0) and presence (v) of inhibitor, as a function of the inhibitor and substrate concentrations, because the equation contains too many parameters. Introducing similar assumptions as in Scheme I:

$$K_{AS} = K'_{AS} = K''_{AS}$$

$$K_{PS} = K'_{PS}$$

$$K_{PI} = K'_{PI}$$

$$k' = k'' = 0$$

Eq. 4 simplifies into Eq. 5, previously derived by Aldridge and Reiner (5, 12):

$$\frac{v\cdot[I]}{v_0 - v} = K_{PI}\left(1 + \frac{[S]}{K_{PS}}\right) \quad (5)$$

No data are available on the numerical values of k'' for ACh or ATCh, but if an inhibitor dramatically reduces the catalytic reaction it is likely that k'' is also much smaller than k. Eq. 5 has the same form as Eq. 2, which describes competition between substrate and inhibitor for the active center. In both Eqs. 2 and 5, the left side is a linear function of $[S]$, but the corresponding intercepts on the abscissa have significantly different values.

If, in Scheme II, the ternary complex IES is not formed (which means that K'_{PI} and K''_{AS} are very large) and if the assumptions made by Aldridge and Reiner (5) are retained:

$$K_{AS} = K'_{AS}$$

$$K_{PS} = K'_{PS}$$

$$k' = k'' = 0$$

Eq. 4 simplifies into:

$$\frac{v\cdot[I]}{v_0 - v} = K_{PI}\left(1 + \frac{[S]}{K_{AS}}\right)\left(1 + \frac{[S]}{K_{PS}}\right) \quad (6)$$

At low substrate concentrations, the ratio $[S]/K_{PS}$ is small, and Eq. 6 resumes the same form as Eq. 2, i.e., the left side of the equation is a linear function of the substrate concentration and the line intersects the abscissa at the value of K_{AS}.

Hunter-Downs plots of $[v/(v_0 - v)]\cdot[I]$ versus $[S]$ were used to evaluate the enzyme-inhibitor and enzyme-substrate dissociation constants. These plots may distinguish between reactions described by Eq. 2 and 5, even though the two equations have the same dependence on the variables I and S. The intercept on the abscissa (K_{PS}) for Eq. 5 corresponds to the substrate inhibition constant K_{SS}, whereas in the case of Eq. 2 the intercept (K_{AS}) corresponds to the substrate Michaelis constant, K_m. The intercepts on the ordinate are equal to the respective enzyme-inhibitor dissociation constants (K_{PI} or K_{AI}). K_m and K_{SS} were also evaluated from pS curves measured in the absence of inhibitor. K_m was estimated from $[S]/v$ versus $[S]$ at $[S] < 1.0$ mM, whereas K_{SS} was estimated from a plot of $1/v$ versus $[S]$ for $[S] > 1.0$ mM.

Lineweaver-Burk plots could not be used for evaluation of dissociation constants, because in our range of substrate concentrations (0.1 to 10.0 mM) they are significantly nonlinear, due to the substrate inhibition. Hunter-Downs analysis of simple competitive or noncompetitive inhibition when the substrate itself is also an inhibitor yields linear plots over a wide range of substrate concentrations. However, because both the substrate and inhibitor are independent variables and the plots relate reaction velocity to the ratio of substrate and inhibitor concentrations, the analysis is limited in distinguishing unique mechanisms.

Results

Kinetic studies. Reversible inhibition of AChE by propidium, haloxon, coumarin, edrophonium, and TMA is shown in Fig. 1. Inhibition was measured with substrate concentrations of up to 10.0 mM, except in experiments with the coumarin derivative, where the highest ATCh concentration was 1.0 mM; at higher ATCh concentrations insufficient inhibition was obtained, and higher coumarin concentrations could not be used due to its low solubility. Inhibition by edrophonium and TMA yielded curvilinear Hunter-Downs plots, whereas linear plots were obtained with propidium, haloxon, and coumarin. For all five inhibitors, the enzyme-inhibitor and enzyme-substrate dissociation constants were calculated from results obtained with ATCh concentrations of up to 1.0 mM; the constants are listed in Table 1.

Propidium and edrophonium have the highest affinities for AChE and yield dissociation constants close to those previously published using different methods (3, 15, 16, 21). The dissociation constants for haloxon, coumarin, and TMA for *Torpedo* AChE are about 3 times greater than reported for erythrocyte AChE (12, 21). Inhibition by TMA was measured in 200 mM buffer and that by the other compounds in 20 mM buffer (Table 1). As noted in several previous studies, dissociation constants for these ligands for AChE are markedly affected by the ionic strength of the medium (Tables 1, 2, and 3).

All ligands compete with AChE at concentrations that yield an enzyme-substrate dissociation constant between 0.24 and 0.49 mM (Table 1). These constants are about 5 times greater than the Michaelis constant for ATCh, but they are 50 times smaller than the substrate inhibition constant (K_{SS}) for ATCh (Table 1). This result is different from that obtained for erythrocyte AChE, where haloxon and coumarin compete with ATCh and ACh at substrate concentrations corresponding to the K_{SS} constants of ATCh and ACh, whereas edrophonium and TMA compete at concentrations corresponding to the K_m of the substrates (12, 21). From the kinetics of inhibition of erythro-

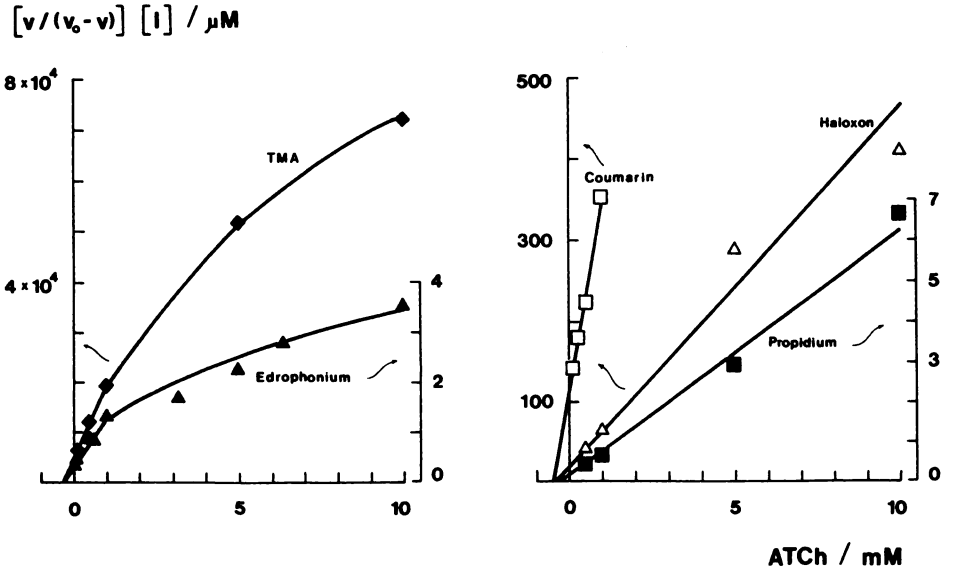

Fig. 1. Inhibition of AChE by the indicated inhibitors measured with ATCh as substrate (Hunter-Downs plots). Inhibitor concentrations were TMA, 5.0–20 mM; edrophonium, 0.10–10 μM; haloxon, 5.0–60 μM; coumarin, 60–120 μM; and propidium, 0.15–15 μM. Each *point* is the mean value of two to five measurements. *Lines* were drawn by eye. *Arrows*, denote the corresponding ordinate.

TABLE 1

Dissociation constants and presumed site-specificity of the ligands for AChE

Data were evaluated from the influence of ligand concentration on the enzyme activity. Constants for ATCh and ACh were evaluated from the linearized pS curves. Constants for the other ligands (measured with ATCh) were taken from the plot shown in Fig. 1, as the intercepts on the ordinate (enzyme-inhibitor constant) or abscissa (enzyme-substrate constant). Shown are the means of two to four experiments. Standard errors are included for three or more measurements.

Ligand	Dissociation constant			Medium
	Enzyme-inhibitor		Enzyme-substrate	
	Active center	Peripheral site		
	μM		mM	
Propidium		0.13	0.24	20 mM $NaPo_4$, pH 7.4
Haloxon		16	0.33	20 mM $NaPO_4$, pH 7.4
Coumarin		116 ± 6	0.49 ± 0.03	20 mM $NaPO_4$, pH 7.4
Edrophonium	0.30 ± 0.06		0.30 ± 0.06	20 mM $NaPO_4$, pH 7.4
TMA	4000 ± 1000		0.26 ± 0.01	200 mM Tris·HCl, pH 8.0

Ligand	Active Center K_m	Peripheral site K_m	Medium
	mM	mM	
ATCh	0.055 ± 0.007	17 ± 3	20 mM $NaPO_4$, pH 7.4
ATCh	0.086 ± 0.010	23 ± 3	200 mM Tris·HCl, pH 8.0
ACh	0.13 ± 0.13	25 ± 13	100 mM NaCl, pH 8.0

TABLE 2

Fluorescent titrations of AChE and DFP-AChE by propidium

Dissociation constants for propidium (K_p) were calculated from Scatchard plots. The number of experiments is shown in parentheses. Means and standard deviations of three or more measurements are given.

Enzyme	Dissociation constant, K_p		
	1.0 mM Tris·HCl, pH 8.0	20 mM $NaPO_4$, pH 7.4	200 mM Tris·HCl, pH 8.0
	μM		
AChE	0.37 (2)		4.0 (2)
DFP-AChE	0.51 ± 0.32 (4)	0.72 ± 0.06 (6)	4.1 ± 3.2 (6)

TABLE 3

Dissociation constants (K_c) for ligands and DFP-AChE

Data were calculated from Eq. 1, using intercepts on the abscissa of the plot shown in Fig. 3 and dissociation constants for propidium given in Table 2. Means and standard deviations are given. The number of experiments is shown in parentheses.

Ligand	Dissociation constant		
	200 mM Tris·HCl, pH 8.0	20 mM $NaPO_4$, pH 7.4	1.0 mM Tris·HCl, pH 8.0
	mM		
ATCh	15 ± 4 (5)		
ACh	24 ± 7 (4)		
TMA	113 ± 31 (3)		
Haloxon		0.022 (2)	0.016 ± 0.010 (5)
Coumarin		0.026 (2)	0.041 ± 0.027 (4)
Edrophonium		0.59 ± 0.20 (3)	

cyte AChE, Aldridge and Reiner (5, 12) suggested that inhibition by haloxon and coumarin occurs at the peripheral site of AChE and that substrate inhibition by ATCh and ACh also occurs at the same peripheral site. The kinetics of inhibition of erythrocyte AChE by haloxon and coumarin yielded constants consistent with Eq. 5, whereas inhibition by edrophonium and TMA yielded constants consistent with Eq. 2. Consequently, the site specificity of the inhibitors for the erythrocyte AChE could be distinguished from the kinetics of inhibition.

This is not the case for the inhibition of *Torpedo* AChE. The kinetics of inhibition by haloxon, coumarin, and propidium are better described by Eq. 2 than by Eq. 5, although propidium is a known peripheral site ligand and haloxon and coumarin are shown in this study to bind to the peripheral site of *Torpedo* AChE. In order to explain this behavior, a theoretical model

was derived (see Eq. 6) that shows that binding of a ligand to the peripheral site might reveal kinetics that cannot be distinguished from kinetics of binding to the active center. Lineweaver-Burk kinetic analysis of competition with propidium for M7C carbamylation (3, 15) also could not distinguish the site of propidium action.

Inhibition of *Torpedo* AChE by edrophonium and TMA showed curvilinear Hunter-Downs plots (Fig. 1). Both compounds are known active center ligands (3, 25), and deviations from linearity were unexpected (see Eq. 2). For TMA it can be shown that binding to the peripheral site in addition to binding to the active center (for equations see Ref. 5) can result in the curvature we observed. This is also indicated by fluorescence titration experiments in this paper. The edrophonium plot is, however, curved more than could be attributed to its significantly weaker binding outside the active center, as shown also by fluorescence titration experiments (see below). A possible explanation is that the affinity of edrophonium for the active center is increased when substrate is bound at the peripheral site, as predicted by Eq. 3.

Fluorescent titrations. Dissociation constants of propidium for native and DFP-modified AChE, determined by direct titration of the enzyme, are given in Table 2. One experiment is shown in Fig. 2. Propidium dissociation constants do not change significantly upon DFP modification of the active center in either low or higher ionic strength medium. This is consistent with reports on methanesulfonylated AChE (3), alkylphosphonyl-AChE (16), and pyrenebutylmethylphosphono-AChE (17, 18). In view of the molecular sizes of the propidium molecule and the modified active center, it seems likely that propidium binds to the modified AChE at a site removed from the active center. This would not necessarily be predicted by the competitive inhibition of ATCh hydrolysis described above, but it does not exclude the mechanism described in Eq. 6. Thus, it is likely that propidium, by virtue of its peripheral site association, changes the active center conformation such that a stabilized enzyme-substrate complex is precluded. In support of this, it can be mentioned that binding of certain ligands to the AChE peripheral site was reported to allosterically affect conformation of the active center (19).

A second means of examining the relationship between substrate and inhibitory ligands is by fluorescence titration. However, because the high turnover number of substrate would preclude maintenance of a constant substrate concentration even if rapid-flow conditions are used, we have modified the AChE active center and then examined the competition between substrates and propidium. The active center modification should not affect propidium binding, as noted above.

The results of propidium back-titration with other ligands are shown in Fig. 3. The enzyme-ligand dissociation constants were calculated from Eq. 1, and the values obtained are given in Table 3. The substrates ATCh and ACh yield dissociation constants similar to the values calculated from kinetic measurements of substrate inhibition (Table 1). The issue might be raised that the effect of ACh and ATCh at high ligand concentrations on propidium dissociation is largely due to the increase in ionic strength contributed by the quaternary salt. This seems unlikely, because the titration with TMA showed a significantly higher dissociation constant than that for either ATCh or ACh (Table 3). Thus, two observations favor a direct competitive titration between either ATCh or ACh and propidium. First, the substrate dissociation constants are in close accord with the respective constants obtained for substrate inhibition. Second, the dissociation constants obtained are much lower than that found for TMA. The contribution due to ionic strength or physical adsorption of the cation on anionic surfaces should be similar for TMA and the substrates. Hence, the different dissociation constants we observe are indicative of discrete site selectivity in binding.

The same approach was used for haloxon and coumarin binding to the DFP-inhibited enzyme. Dissociation of propidium from its binding site indicates that binding to the peripheral site is inherent to their mode of action. A comparison between dissociation constants for haloxon and coumarin derived from back-titration of propidium (Table 3) with those derived from kinetic measurements (Table 1) shows that, for both compounds, the two constants are in good agreement. This, in turn, indicates that the direct titration and inhibition

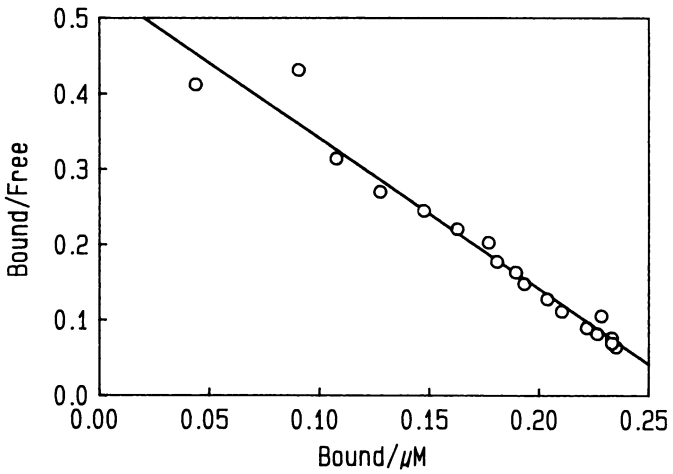

Fig. 2. Scatchard plot for titration of DFP-AChE by propidium in 1.0 mM Tris·HCl buffer, pH 8.0. Descriptions of the axes refer to concentrations of propidium, bound on the enzyme or free in solution. One experiment is shown. The *line* was calculated by linear regression.

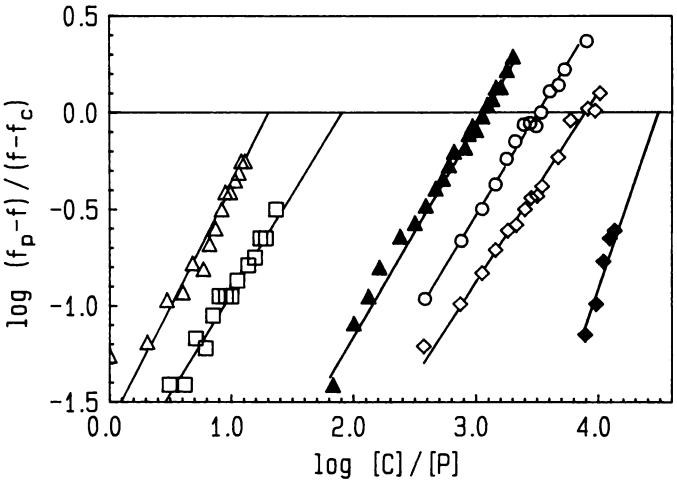

Fig. 3. Back-titration of propidium bound to DFP-AChE. Ligands were haloxon (△) (10–150 μM), coumarin (□) (10–250 μM), edrophonium (▲) (0.40–28 mM), ATCh (○) (10–230 mM), ACh (◇) (10–291 mM), and TMA (◆) (19–165 mM). Medium was 20 mM $NaPO_4$ buffer, pH 7.4, containing 2–5% ethanol, for haloxon and coumarin, 20 mM $NaPO_4$ buffer, pH 7.4, for edrophonium, or 200 mM Tris·HCl buffer, pH 8.0, for ATCh, ACh, and TMA. Single experiments are shown. *Lines* were calculated by linear regression.

kinetics involve the same site on the enzyme. Modification of the active center by DFP or even larger organophosphates does not affect binding of propidium (Table 2) (3, 15–18). The same obviously should hold for haloxon and coumarin, thus confirming that both inhibitors bind to the peripheral site. Haloxon probably interacts with the peripheral site by virtue of its coumarin moiety, indicating that uncharged ligands may also bind to a peripheral site. This secondary peripheral site association distinguishes haloxon from most other organophosphates by its exhibition of altered kinetics of the active center phosphorylation, as was shown for the erythrocyte AChE (5, 12, 21). Another aliphatic organophosphate was shown recently to exhibit binding at two sites (26).

Displacement of propidium from the peripheral site by edrophonium or TMA requires ligand concentrations that are 2 to 3 orders of magnitude higher than their dissociation constants derived from kinetic measurements (compare Tables 1 and 3), thus confirming that the AChE active center is the major site of their association. Complete dissociation of propidium by haloxon, coumarin, and TMA was not achieved (Fig. 3), owing to the low solubility of haloxon and coumarin and the low affinity of TMA. These limitations were not encountered with edrophonium, ACh, and ATCh.

Discussion

This rather straightforward correlative analysis of kinetic constants of inhibition with the capacity of the same ligands to dissociate propidium from its site of occupation brings several issues to light. First, inhibitors acting at either the active center or the peripheral site can, under certain conditions, yield competitive inhibition kinetics. This was previously indicated for propidium (3), where uncompetitive kinetics for inhibition of M7C carbamylation of the enzyme were noted at low ionic strength (1 mM Tris), whereas at higher ionic strength (0.1 M NaCl, 40 mM $MgCl_2$) inhibition appeared competitive. As the ionic strength is raised, the dissociation constant for propidium increased (0.3 versus 3.3 μM); nevertheless, the values obtained from the inhibition kinetics were in good accord with those obtained by direct titration (3). Analogous behavior is evident when propidium inhibition was compared with that of two analogues, hexidium and decidium, where six and 10 methylene groups connect the endocyclic and exocyclic quaternary groups (15). We find a similar situation here, where propidium inhibition of ATCh hydrolysis appears competitive, yet propidium is binding to a peripheral site.

Second, we have provided evidence that the substrate inhibition site for ACh and ATCh is the peripheral anionic site defined by the binding of propidium and certain other ligands. By phosphorylating the active center so that inhibition is complete, these substrates may be employed to back-titrate propidium competitively from the peripheral site. We have found that the constants obtained in the competitive back-titrations are in close accord with the substrate inhibition constants. Moreover, we found a higher dissociation constant for TMA than for the natural substrate ACh or its thioester analogue. If propidium dissociation were being affected by entry of the cation to the phosphorylated active center, then we would have anticipated that TMA would be more effective than the acylcholine analogues in entering the already acylated active center. The fact that the larger ACh analogues have the lower dissociation constants points to a site-specific action, and this site is not the active center. Similarly, substrate inhibition occurs at this peripheral site and allosterically affects active center conformation. This, in turn, could affect either the acylation or deacylation rates.

Third, we have extended our approach to examine whether complex inhibition kinetics of some organophosphates can be explained on the basis of a secondary interaction with the peripheral site. In fact, this can be demonstrated for haloxon and its leaving group coumarin. It was earlier shown that the ability of haloxon to phosphorylate the active center was affected by its reversible binding to a substrate inhibition site (5, 12, 21). By analogy, binding of excess substrate to the peripheral site in substrate inhibition may affect acylation of the active center, or its deacylation rate, and thus inhibit further substrate hydrolysis.

Although the lower haloxon affinity makes the *Torpedo* enzyme less than ideal for demonstrating anomalous haloxon inhibition kinetics shown for the erythrocyte enzyme (12, 21), the low abundance of erythrocyte enzyme makes it difficult to obtain sufficient quantities of the purified entity for the fluorescent titrations. The differences in haloxon and coumarin inhibition kinetics for the two enzymes suggest that *Torpedo* AChE is more sensitive to ionic strength changes in the formation of ternary complexes with substrates and haloxon or coumarin than is erythrocyte AChE.

The role of substrate inhibition in affecting the residence time of ACh in the synapse can only be speculated upon. For substrate inhibition to be significant, released ACh within the synapse in the vicinity of AChE would have to approach concentrations of several millimolar. Estimates of synaptic concentrations of ACh are in the range of 0.3 mM (27), and it is possible that, if we are examining diffusion from a vesicle or a discrete release site, transient concentrations of ACh at the point source of release would be transiently higher than the overall estimates of synaptic ACh. A diminished catalytic efficiency of AChE at high concentrations of substrate would serve to prolong transiently the residence time of ACh in the synapse. This in turn may allow ACh diffusing from a point source, to envelop a larger surface by the time it reaches the receptor on the postsynaptic membrane. Because much of the synaptic AChE in the neuromuscular junction is in the basal lamina rather than on the plasma membrane, its position may be more proximal to the ACh diffusing from a point source.

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