IDENTIFICATION OF A MODIFIER SITE ON ACETYLCHOLINESTERASE WITH AFFINITY FOR d-TUBOCURARINE*

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Received June 23,1972

Summary. Decamethonium and d-tubocurarine displace N-methylacridinium ion, a potent fluorescent inhibitor of acetylcholinesterase, from the surface of the enzyme. Decamethonium is competitive with N-methylacridinium which indicates that the binding sites for these ligands overlap. However, the displacement of N-methylacridinium ion by d-tubocurarine requires the existence of a binding site for d-tubocurarine in addition to the active site. Since the affinities for d-tubocurarine at both sites are comparable, two well defined ligand binding sites must exist for each catalytic site that is titratable by 7-dimethylcarbamyl-N-methylquinolinium iodide.

Kinetic studies have suggested that acetylcholinesterase (AChE) from cholinergic tissue possesses a ligand binding site remote from the active site of the enzyme (1-3). Since alternative interpretations of the kinetic data are possible (4), we have investigated the binding site topography of AChE with fluorescent ligands to see if any further evidence could be obtained for the existence of peripheral binding sites specific for cationic ligands.

N-Methylacridinium ion (I) was the fluorescent ligand used in this work since previous studies have indicated that it binds very tightly and exclusively to the active site of AChE, and that its intense fluorescence in aqueous solution is strongly quenched upon binding to the enzyme surface (5). Since the free and bound concentrations of N-methylacridinium ion can be readily monitored in solutions containing this ligand and AChE, it is possible to measure the binding of non-fluorescent inhibitors to AChE by observing

* Supported by USPHS Grant No. AM-12789 and USPHS DE-89. Computing assistance was obtained from the Health Science Computing Facility, UCLA, sponsored by NIH Special Resources Grant RR-3.

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the fluorescence increase due to the displacement of N-methylacridinium ion as increasing amounts of other ligands are added.

In the present communication, the binding of decamethonium (II) and d-tubocurarine (III) to AChE has been examined by studying the displacement of N-methylacridinium ion from the enzyme surface. Our results clearly demonstrate the existence of a specific ligand binding site remote from the active site which has a high affinity for the pachycurare, d-tubocurarine.

Materials and Methods. Acetylcholinesterase from Electrophorus electricus was obtained from Worthington Biochemical Corp. Active site normality was measured by titration with 7-dimethylcarbamyl-N-methylquinolinium iodide (5). N-Methylacridinium iodide (I) was prepared as previously reported. Decamethonium chloride (II) was prepared by eluting decamethonium bromide (K&K Laboratories, Plainview, N.Y.) through a Dowex-1 chloride column with water. d-Tubocurarine (III) was obtained from K&K and recrystallized from 0.1 N HCl prior to use.

Fluorescence measurements were performed with an Aminco-Bowman Spectrophotofluorometer equipped with a ratio photometer and a thermostatted cell compartment. All experiments were done at 25° in 0.001 M phosphate buffer, pH 7.0, to which 0.001 M dihydroquinone was added to stabilize the fluorescence of I ($\lambda_{excit.}$ = 360 nm, $\lambda_{emis.}$ = 490 nm). In each experiment, several aliquots (ranging in volume from 2-10 µl) of the displacer (II or III) were added to 1.0 ml solutions containing approximately 1 x 10^{-7} N of both I and AChE. After the addition of each aliquot, the fluorescence increase was recorded. The resulting displacement pattern of I was then plotted as the free concentration of I as a function of log [displacer]. A nonlinear least squares regression analysis was performed on a 360/91 IBM computer to determine which of the various models indicated below corresponded best to the experimental curve obtained.

Results and Discussion. Three simple models were considered as reasonable possibilities to describe the displacement of I from the enzyme. All

of the models tested incorporate a single binding site for I on the enzyme (Site 1) which has previously been shown to be the anionic subsite of the active site (5). A second ligand binding site (Site 2) distinct from the active site is incorporated in Models ii and iii. Displacing ligands (decamethonium and d-tubocurarine) are indicated generally as D, and the subscripts associated with D refer to the site at which the displacing ligand is binding and from which it dissociates. The subscripts associated with the dissociation constants refer to the species into which a given complex dissociates.

Model i - The displacing ligands compete with I for Site 1 as in eqs. 1 and 2.

$$E + I \rightleftharpoons E\text{-}I \qquad K_{E,I} = \frac{(E)(I)}{(E\text{-}I)} \tag{1}$$

$$E + D \rightleftharpoons E\text{-}D_1 \qquad K_{E,D_1} = \frac{(E)(D)}{(E\text{-}D_1)} \tag{2}$$

Model ii - The displacing ligands bind only at Site 2 but not at Site 1 so that a ternary complex with composition $E\text{-}I\text{-}D_2$ forms and eqs. 1,3,4,5 apply.

$$E + D \rightleftharpoons E\text{-}D_2 \quad K_{E,D_2} = \frac{(E)(D)}{(E\text{-}D_2)} \tag{3}$$

$$EI + D \rightleftharpoons E\text{-}I\text{-}D_2 \quad K_{E\text{-}I,D_2} = \frac{(E\text{-}I)(D)}{(E\text{-}I\text{-}D_2)} \tag{4}$$

$$E\text{-}D_2 + I \rightleftharpoons E\text{-}I\text{-}D_2 \qquad K_{E\text{-}D_2,I} = \frac{(E\text{-}D_2)(I)}{(E\text{-}I\text{-}D_2)} \tag{5}$$

Model iii - The displacing ligand competes with I for Site 1 and also binds to Site 2 so that an additional ternary complex $E\text{-}D_1\text{-}D_2$ forms and eqs. 1-7 apply.

$$E\text{-}D_2 + D \rightleftharpoons E\text{-}D_1\text{-}D_2 \qquad K_{E\text{-}D_2,D_1} = \frac{(E\text{-}D_2)(D)}{(E\text{-}D_1\text{-}D_2)} \tag{6}$$

$$E\text{-}D_1 + D \rightleftharpoons E\text{-}D_1\text{-}D_2 \qquad K_{E\text{-}D_1,D_2} = \frac{(E\text{-}D_1)(D)}{(E\text{-}D_1\text{-}D_2)} \tag{7}$$

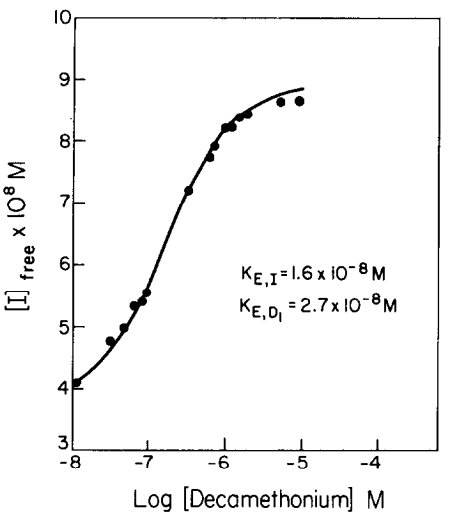

Fig. 1.

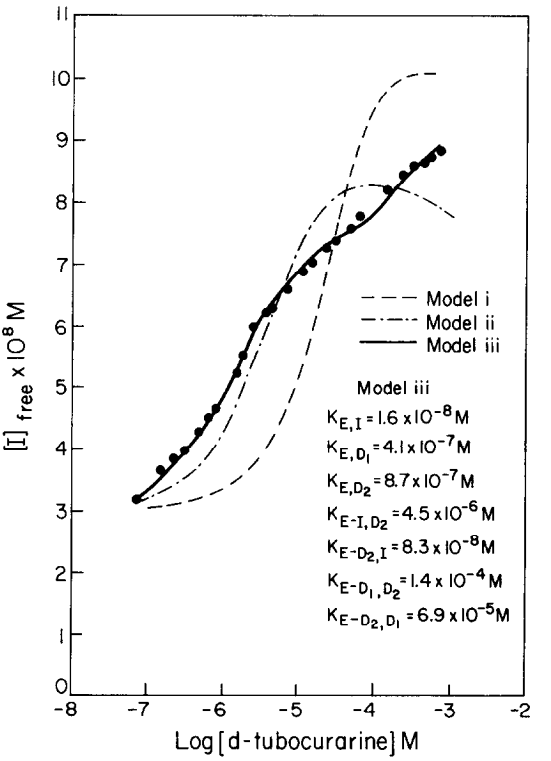

Fig. 2.

Fig. 1. The displacement of N-methylacridinium ion (I) by decamethonium (II). Conditions are described in Materials and Methods. Total I and AChE were initially 1.0 x 10^{-7} M and 9.0 x 10^{-8} N respectively. There was an 11% volume dilution by the end of the experiment from the cumulative aliquots of II added. The solid line is the best fit of the data using a non-linear least squares analysis based on Model i. The value of $K_{E,I}$ was determined independently and supplied to the regression program.

Fig. 2. The displacement of N-methylacridinium ion (I) by d-tubocurarine (III). Conditions are described in Materials and Methods. Total I and AChE were initially 1.5 x 10^{-7} M and 1.8 x 10^{-7} N, respectively. There was a 12% volume dilution by the end of the experiment from the cumulative aliquots of III added. $K_{E,I}$ was determined independently and supplied to the regression program.

The patterns of displacement of N-methylacridinium ion (I) from acetylcholinesterase by decamethonium (II) and d-tubocurarine (III) are shown in Figs. 1 and 2, respectively. The results presented in Fig. 1 clearly demonstrate that Model i accurately describes the mode of displacement of N-methylacridinium ion by decamethonium and suggests these two ligands have

overlapping binding sites. The close agreement of the dissociation constant for II, derived from the data in Fig. 1 ($K_{E,D_1} = 2.7 \times 10^{-8}$ M), and the inhibition constants for II obtained from steady state kinetics with acetylthiocholine ($K_I = 3.5 \times 10^{-8}$ M) further support the validity of Model i. Models ii and iii also provide a fit comparable to Model i but all the dissociation constants involving ternary complexes become infinitely large. These more complex models therefore become indistinguishable from the more simple Model i.

The d-tubocurarine displacement curve (Fig. 2), on the other hand, is best described by Model iii. Both Model i and Model ii give a least squares fit to the data which is clearly unacceptable. These curves converge on a residual sum of squares which is over 20 times that obtained with Model iii. The dissociation constants for the binary and ternary complexes in Model iii are reported in the insert in Fig. 2 and represent the averages of five experiments.

Two important conclusions can be drawn from the relative magnitude of these dissociation constants. First, the peripheral site must be a well defined ligand binding site since the constant describing the dissociation of d-tubocurarine from the active site, Site 1 ($K_{E,D_1} = 4.1 \times 10^{-7}$) is only one half that for the peripheral site, Site 2 ($K_{E,D_2} = 8.7 \times 10^{-7}$ M). Second, the affinity of both N-methylacridinium ion and d-tubocurarine for the active site ($K_{E\text{-}D_2,I}$ and $K_{E\text{-}D_2,D_1}$) is reduced when the peripheral site is occupied by d-tubocurarine (i.e., compare $K_{E\text{-}D_2,I}$ to $K_{E,I}$ and $K_{E\text{-}D_2,D_1}$ to $K_{E\text{-}D_1}$). Since binding to the peripheral site (Site 2) can weaken the affinity of inhibitors at the active site, the binding of substrates at the active site is probably affected similarly. A further consequence of binding at Site 2 may be alterations in the rate of acylation and deacylation of AChE by acetylcholine and various pseudosubstrates.

The direct demonstration of a peripheral binding site presented here supports the suggestions based on kinetic (1, 2), nuclear magnetic resonance

(6) and chemical modification studies (3) that a peripheral binding site exists which is topographically distinct from the active site. The physiological importance of this peripheral binding site cannot be deduced from the data presently available. As a first step in assessing its possible significance, its specificity for other cholinergic ligands will be examined by using N-methylacridinium ion and a bifunctional fluorescent probe, bis-(3-amino-pyridinium)-1,10 decane diiodide (5). Both these fluorescent ligands may also be useful in studying the recently isolated neurotoxin-binding protein (7, 8, 9) and other proteins with binding specificity for cholinergic ligands (10, 11).

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