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Conference Paper

Interaction of Ligand Binding Sites of Acetylcholinesterase*

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Ligand binding properties of acetylcholinesterase from *Electrophorus electricus* have been investigated under equilibrium and transient state conditions with the reversible fluorescent probes, *N*-methylacridinium (*N*-MAC) and bis(3-aminopyridinium)-1,10-decane (DAP). *N*-MAC binds at the active site and DAP, an analog of decamethonium, bridges the active site and peripheral modifier site. The probes were used to monitor the binding of ligands that interact at one or both of these sites with resultant probe displacement. Using a nonlinear least-squares analysis to reduce fluorescent probe displacement data, *d*-tubocurarine was found to bind at a site remote from the active site. In physiological ionic strength media, *d*-tubocurarine binds exclusively at the peripheral modifier site, and in low ionic strength media it binds significantly to both the peripheral site and active site. In both ionic strength media, the peripheral site was found to be the same site that interacts with the second cationic function of bis-quaternary ammonium compounds like decamethonium and DAP. When *d*-tubocurarine occupies the peripheral site, the affinity of the enzyme for active site ligands is decreased. There is a positive correlation between the size of the active site ligand and the degree of *d*-tubocurarine induced destabilization suggesting steric factors may be operative. Gallamine binding is mutually exclusive with active site ligands. Rapid mixing stopped flow experiments were used to determine if this results from gallamine binding at the active site or binding at the peripheral site with resultant conformational effects mediated to the active site. The demonstration of a transient gallamine—enzyme—*N*-MAC ternary complex suggests the latter binding pattern occurs.

Acetylcholinesterase (AcCh-esterase)***, solubilized from cholinergic membranes, may be more intimately involved in the events of synaptic transmission than strictly serving to hydrolyze released acetylcholine (AcCh) subsequent to depolarization of the post-synaptic membrane. In support of this, there have

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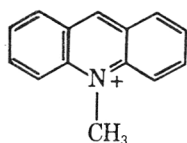
** National Institutes of Health, Career Development Awardee

*** Abbreviations used are: AcCh-esterase, acetylcholinesterase; AcCh, acetylcholine; AcSCh, acetylthiocholine; *N*-MAC, *N*-methylacridinium; DAP, bis(3-aminopyridinium)-1,10-decane.

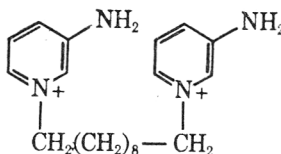
been a number of reports implicating the presence of a non-catalytic ligand binding site on AcCh-esterase remote from the active site¹⁻², which, when occupied, markedly affects ligand affinity and probably catalysis at the active site.

Experiments aimed at understanding the functional significance, if any, of the peripheral site through characterizing its specificity and interaction with the active site have been hindered by experimental limitations in most approaches. The sole use of kinetic criteria has been complicated by the two step kinetic mechanism of AcCh-esterase and the preference of both sites for cationic ligands¹⁻⁸. The application of affinity labeling⁹⁻¹¹ and n.m.r. techniques¹² have been valuable in identifying the peripheral site, but their inherent limitations have prevented thorough characterization.

In order to examine the peripheral modifier site and its interactions with the probes themselves have been defined through direct fluorimetric titration parameters, reversible fluorescent probes were prepared to monitor binding of various cholinergic ligands of interest to the two sites. Two compounds, *N*-methylacridinium (*N*-MAC) and bis(3-aminopyridinium)-1,10-decane (DAP), an analog of decamethonium, were found to be particularly valuable in this respect in studies with AcCh-esterase from *Electrophorus electricus*^{4,5,13}, and more recently with the AcCh-receptor (14) and serum cholinesterase¹⁵.



N-MAC



DAP

Both fluorescent probes have a strong affinity for AcCh-esterase and, more importantly, their intense fluorescence in aqueous solution is greatly quenched when bound to the enzyme surface¹³. The binding properties of the probes themselves have been defined through direct fluorimetric titration and inhibition kinetics. Both compounds bind to AcCh-esterase with a 1:1 stoichiometry relative to the active site. *N*-MAC binds exclusively to the anionic subsite of the active site and DAP, with two cationic functions separated by a ten carbon alkyl chain, bridges the two sites with one cationic group binding at the active site and the other at the peripheral site. Since the concentrations of the free and bound forms of *N*-MAC and DAP can be measured in a solution containing probe and enzyme, they can serve in competitive equilibrium studies and transient state rapid mixing experiments to monitor the binding properties of any compound that can cause partial or total probe displacement.

Using this approach, it has been possible to more clearly define the specificity of the peripheral site and its interactions with the active site. Of particular interest is the observation that the peripheral site, while preferring cationic ligands similar to those that bind at the active site, is the exclusive site of binding of *d*-tubocurarine and probably gallamine under physiological ionic strength conditions. Binding to the peripheral site greatly decreases ligand

affinity and, perhaps, catalytic efficiency at the active site. In contrast to physiological ionic strength conditions, in low ionic strength media, *d*-tubocurarine binds to both the active site and peripheral site demonstrating solubilized AcCh-esterase can exist in at least two functionally distinct forms in solution^{4,5}.

The equilibrium experiments involving the displacement of fluorescent probes from the enzyme by cholinergic ligands which, in part, lead to these conclusions, were examined in respect to three plausible ligand binding models (Table I). In the simplest scheme, model (i), the displacing ligand binds at

TABLE I.

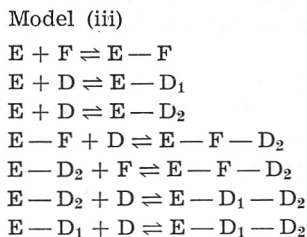
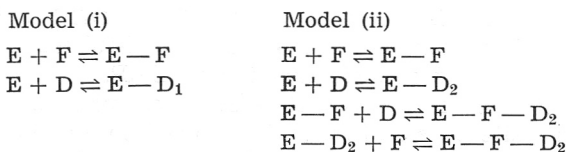
Acetylcholinesterase ligand binding models assuming one fluorescent probe binding site and one or two displacing ligand binding sites

	Binding site of displacing ligand	
	Fluorescent probe site	Remote site
Model (i)	+	—
Model (ii)	—	+
Model (iii)	+	+

the fluorescent probe binding site. The relationship is strictly competitive with binding of the two ligands mutually exclusive. Model (ii) is a partially competitive scheme where the displacing ligand, probe and enzyme can form a ternary complex. This model would be applicable, for example, if the competing ligand bound to the peripheral site and weakened the affinity of *N*-MAC at the active site.

Model (iii) is the most inclusive and is applicable when the competing ligand can bind at both a remote site and the fluorescent probe binding site. In this situation a ternary complex of competitor—probe—enzyme may form as well as competitor—competitor—enzyme.

The equilibria involved in the three models are given below, where E is the enzyme, F in the fluorescent probe and D is the displacing ligand, which can bind at either the fluorescent probe site, D₁, or a remote site, D₂.



The mathematical equations defining model (iii) are too complex for standard graphical analysis, which necessitated the use of computer-assisted non-linear regressions to evaluate the consistency of experimental data with the three models.

Three cholinergic effectors (decamethonium, *d*-tubocurarine and gallamine) were examined as displacing ligands using *N*-MAC and DAP as fluorescent probes in both physiological (0.1 M NaCl, 0.02 M MgCl₂, 0.01 M phosphate, pH = 7.0) and low (0.001 M NaCl, 1.0×10^{-4} M MgCl₂, pH = 7.0) ionic strength media. A summary of the results is presented in Table II. Note that each of the models was found to be operative in at least one instance.

TABLE II.

Models best describing the data for N-MAC and DAP displacement by cholinergic ligands

Displacing Ligand	Fluorescent Probe	Model Best Describing Data	
		Physiological ^a Strength	Low Ionic ^b Strength
Decamethonium	<i>N</i> -MAC	(i)	(i)
	DAP	(i)	(i)
Gallamine	<i>N</i> -MAC	(i)	(i)
	DAP	(i)	(i)
<i>d</i> -Tubocurarine	<i>N</i> -MAC	(ii)	(iii)
	DAP	(i)	(iii)

^aMedia contained 0.1 M NaCl, 0.02 M MgCl₂, and 0.01 M phosphate, pH = 7.0.

^bMedia contained 0.001 M NaCl and 2.0×10^{-4} M MgCl₂, pH = 7.0.

Under physiological ionic strength conditions, decamethonium, gallamine and *d*-tubocurarine were competitive with DAP binding [model (i)] suggesting the binding site of each of these ligands overlaps that of DAP. Similarly, using *N*-MAC as the fluorescent probe, decamethonium and gallamine were competitive. However, *d*-tubocurarine displacement of *N*-MAC was most consistent with model (ii) (Figure I), which requires the formation of a *d*-tubocurarine—*N*-MAC-enzyme complex. Since *N*-MAC is known to bind at the active site, *d*-tubocurarine must bind elsewhere. The location of this site is suggested by the DAP displacement results. Since DAP and *d*-tubocurarine are competitive, it is reasonable to conclude that *d*-tubocurarine binds at the site specific for the second cationic function on DAP.

The destabilization of the *N*-MAC-enzyme complex with *d*-tubocurarine at the peripheral site is significant. A comparison of the *N*-MAC-enzyme dissociation constant in the presence and absence of *d*-tubocurarine shows about a six-fold increase when *d*-tubocurarine is occupying the peripheral site (Table III).

The ligand binding parameters determined at low ionic strength differ both quantitatively and qualitatively from those at physiological ionic strength. There is a marked increase in ligand affinity at low ionic strength, consistent with kinetic studies and attributable to an increase in Coulombic forces. The binding patterns of gallamine and decamethonium remained competitive with both *N*-MAC and DAP as observed at physiological ionic strength, but *d*-tubo-

curarine, rather than binding exclusively at the peripheral site, binds at both the peripheral site and active site. Figure 2 illustrates the data obtained from displacement of *N*-MAC by *d*-tubocurarine at low ionic strength. Clearly,

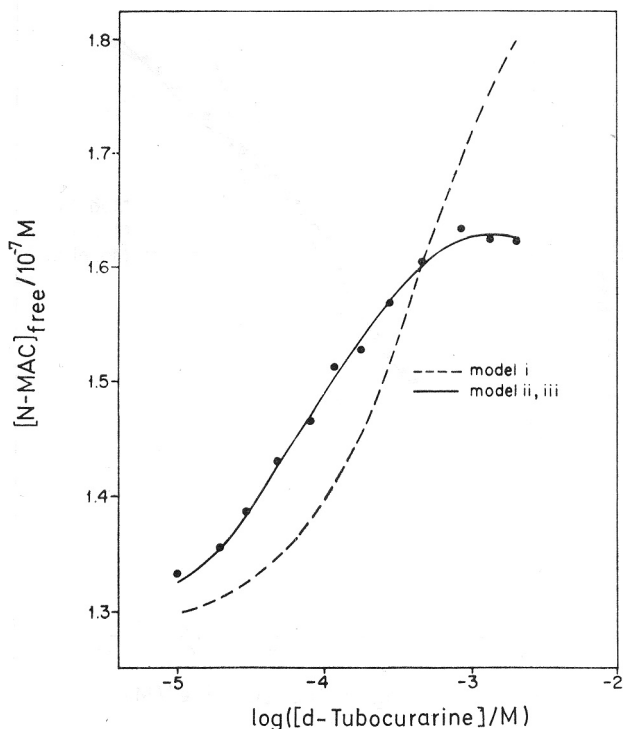


Fig. 1 — *d*-Tubocurarine displacement of *N*-MAC at physiological ionic strength. Solid dots are the experimental points. In the regression analysis, model (iii) was reduced and became equivalent to model (ii).

TABLE III.

Destabilization of active site ligands by d-tubocurarine binding to the peripheral site on acetylcholinesterase

Assay Conditions	Free Enzyme ^a	Enzyme- <i>d</i> -Tubocurarine ^a	Enzyme- <i>d</i> -Tubocurarine ^b Free Enzyme
Physiological ionic strength (0.16)	K_{N-MAC}/M		
	2.5×10^{-7}	1.5×10^{-6}	6.0
Low ionic strength (0.001)	1.5×10^{-8}	8.3×10^{-8}	5.5
Low ionic strength (0.001)	$K_{d-tubocurarine}/M$		
	4.1×10^{-7}	6.9×10^{-5}	168

^aDissociation constant for *N*-MAC or *d*-tubocurarine binding at the active site with the peripheral site either unoccupied (free enzyme) or bound with *d*-tubocurarine (enzyme-*d*-tubocurarine).

^bRatio of dissociation constants for active site ligands with the peripheral site free and bound with *d*-tubocurarine.

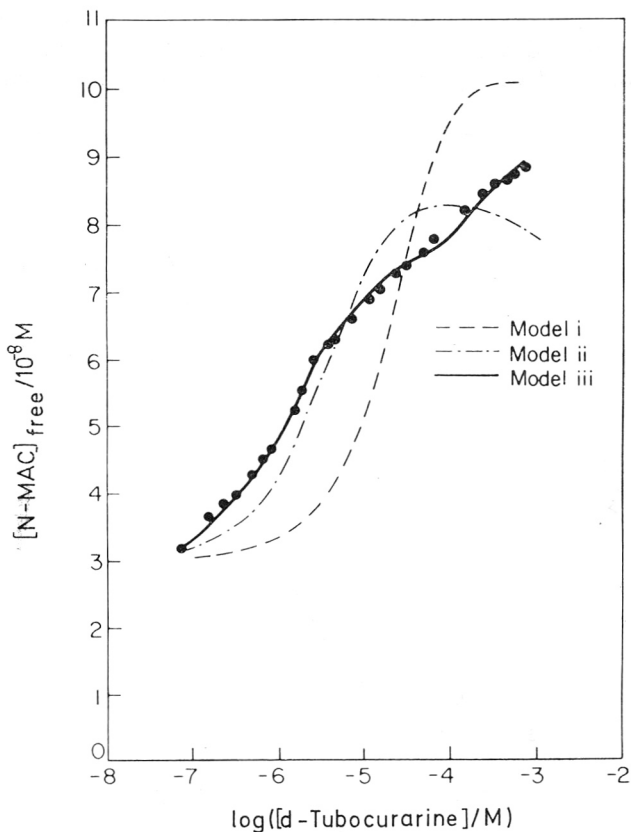


Fig. 2 — *d*-Tubocurarine displacement of *N*-MAC at low ionic strength. Solid dots represent experimental points, and the lines are best fits generated in non-linear regression analyses using the various models.

model (iii) is the best description of the data. Both sites have strong and comparable affinities for *d*-tubocurarine. The dissociation constant at the active site is 4.1×10^{-7} M and at the peripheral site is slightly weaker, 8.7×10^{-7} M. The destabilization of the *N*-MAC enzyme complex with *d*-tubocurarine bound at the peripheral site is similar to that observed at physiological ionic strength (Table III), or about six-fold weaker in the presence of *d*-tubocurarine than in its absence.

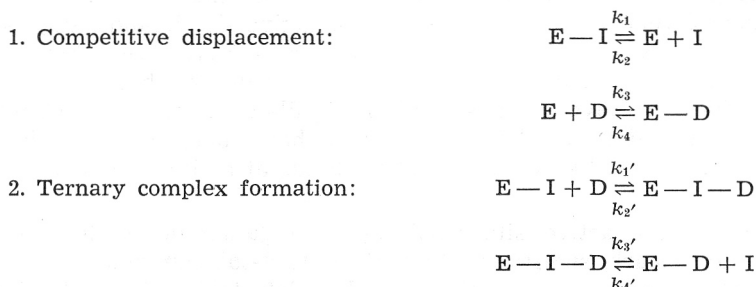
Similar results were obtained using DAP as the fluorescent probe. The data was most consistent with model (iii), permitting a *d*-tubocurarine-*d*-tubocurarine—enzyme complex; however, unlike the *N*-MAC pattern, a ternary complex could not form with DAP, *d*-tubocurarine and enzyme. This is again consistent with the postulate that the sites that bind the two moles of *d*-tubocurarine at low ionic strength are the same sites that interact with the two cationic groups on DAP.

The effects on active site ligands caused by *d*-tubocurarine binding to the peripheral site may reflect steric factors, induced protein conformational changes or both. The distance between the two sites, based on the separation

between the cationic functions of DAP, is 14 Å or less, depending on the degree of extension of the connecting alkyl chain. The relative affinities of compounds in the polymethonium series, however, suggest that decamethonium binds in nearly an extended conformation¹⁶. The proximity of the two sites indicates steric factors may be important since *d*-tubocurarine will span most of the distance if the bulk of the molecule is directed in the region between the two sites. This possibility is supported by the observation that with *d*-tubocurarine bound at the peripheral site, the destabilization of the second molecule of *d*-tubocurarine binding at the active site is much greater than the destabilization of *N*-MAC at the active site (Table III). If steric factors are significant it might be anticipated that the effects would be more significant when comparing the bulkier *d*-tubocurarine to *N*-MAC.

On the other hand, ligand induced conformational changes should not be excluded. *O. r. d.* changes have been demonstrated subsequent to 3-hydroxydimethylethylammonium binding to AcCh-esterase¹². Many of the kinetic results which require a modifier site can be rationalized by steric considerations if the modifier site is near the active site. However, kinetic studies which demonstrate ligand induced effects on rates of acylation or deacylation implicate protein conformational changes^{2,3}. Kitz *et al.*³ report gallamine can accelerate decarbamylation of the dimethylcarbamylyl enzyme at concentrations which do not affect AcCh hydrolysis. Clearly, if the active site were involved in the gallamine induced acceleration mechanism, the rate of AcCh hydrolysis would be altered. These observations provide evidence that ligand binding to a remote site can modulate catalytic efficiency as well as ligand affinity at the active site.

Although steady-state kinetic studies have indicated that gallamine, an antagonist with pharmacological effects similar to *d*-tubocurarine, binds to a site remote from the active site, the results of the fluorescent probe displacement experiments presented here indicate that gallamine is strictly competitive with *N*-MAC. In order to help resolve this ambiguity, the rates of displacement of *N*-MAC from the enzyme by gallamine were examined by stopped flow techniques. The rationale for these experiments was that the net displacement of *N*-MAC from the enzyme can proceed by the two mechanisms indicated below.



The first mechanism would be operative if the binding sites of *N*-MAC and gallamine shared a topographically identical region. If the binding site of the displacing ligand (D or gallamine) overlapped that of the probe (I or *N*-MAC) then the probe must dissociate prior to the binding of the displacing ligand. When conditions are used which assure that binding of the displacing ligand

is not rate limiting, the rate of dissociation of fluorescent probe will be k_1 , or the off-rate characteristic of the enzyme—probe binary complex. The latter rate constant can be determined independently.

The second mechanism would be expected if the binding site of the probe and displacing ligand do not overlap. In this case, a transient ternary complex of probe-enzyme—displacing ligand will form, and the probe dissociates from a ternary complex. This rate should be different from the rate of dissociation from the binary complex.

The results of experiments using several cholinergic ligands to displace *N*-MAC from the active site are reported in Table IV. The half-lives for the

TABLE IV.

Rates of Displacement of N-Methylacridinium ion from acetylcholinesterase^a

Displacing Ligand	Concentration/M	Half Life/ms
Choline chloride	5.0×10^{-3}	5.7
Decamethonium	5.0×10^{-6}	6.7
	2.5×10^{-5}	7.5
<i>d</i> -Tubocurarine	5.0×10^{-5}	26.1
	2.5×10^{-4}	44.0
Atropine	5.0×10^{-3}	21.5
Gallamine	7.5×10^{-5}	12.8

^aMedia contained 0.001 M phosphate, pH = 7.0, 25 °C.

displacement of *N*-MAC by known active site ligands, such as decamethonium and choline, are equal to the half-life of *N*-MAC dissociation from the binary complex determined independently in rapid dilution experiments. This implicates mechanism 1, since the dissociation of *N*-MAC must occur prior to the binding of the displacing ligand. On the other hand, the rate of *N*-MAC displacement by *d*-tubocurarine and atropine is significantly slower than that observed for active site ligands. These results are consistent with the mechanism requiring prior ternary complex formation. Gallamine also shows a rate which is perturbed relative to that observed for active site ligands, suggesting it can form a ternary complex with enzyme and *N*-MAC even though the complex is unstable and not detectable by equilibrium methods. Therefore, transient state kinetics have demonstrated that gallamine and *N*-MAC, which appear competitive under equilibrium conditions, have topographically distinct sites, and that gallamine, like *d*-tubocurarine, binds at a site remote from the active site.

The effects at the active site resulting from gallamine binding to the peripheral site are clearly significant. In contrast to *d*-tubocurarine, no ternary complex of *N*-MAC—enzyme—displacing ligand is detectable with gallamine at equilibrium. The destabilization of the active site ligand, *N*-MAC, appears to be at least an order of magnitude greater when gallamine is occupying the peripheral site compared to *d*-tubocurarine.

In summary, the application of *N*-MAC and DAP to monitor binding at the various ligand binding site on AcCh-esterase under equilibrium and trans-

ient state condition has lead to the clear identification of a ligand binding site approximately 14 Å from the active site. The remote site has a unique specificity and prefers large polycationic ligands like *d*-tubocurarine, atropine and probably gallamine (Fig. 3). Significant interaction between the active

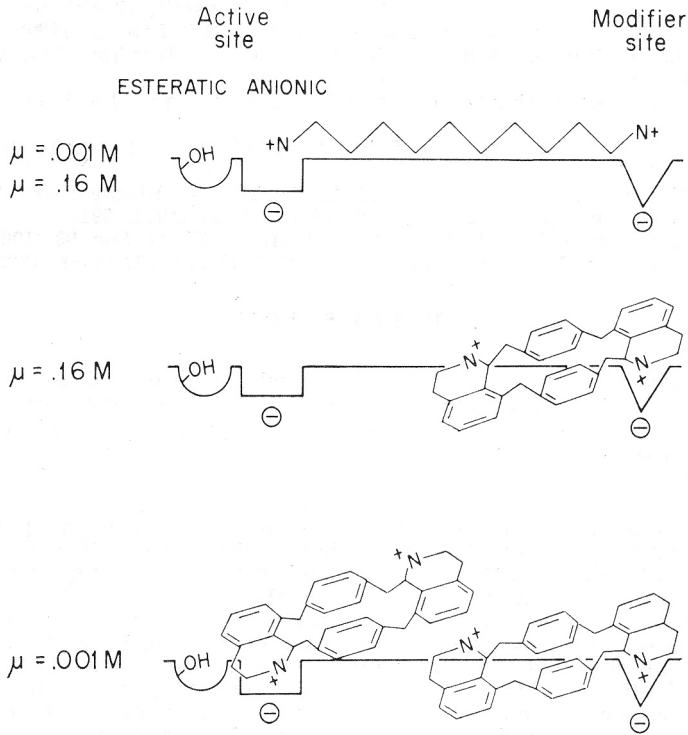


Fig. 3 — Schematic representation of acetylcholinesterase ligand binding topography with decamethonium and *d*-tubocurarine bound under physiological and low ionic strength conditions.

site and peripheral site can be demonstrated. When the peripheral site is occupied, there is a resultant decrease in ligand affinity and possibly catalytic efficiency at the active site. Interestingly, the enzyme can exist in two ionic strength dependent forms as judged by *d*-tubocurarine binding. Under physiological ionic strength conditions AcCh-esterase binds *d*-tubocurarine exclusively at the peripheral site, while under low ionic strength conditions, there is significant binding at both the cationic site and peripheral site. The form of the enzyme which most accurately approximated the membrane bound enzyme remains to be determined.

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DISCUSSION

P. W. Taylor:

I was surprised that the half-life of *N*-methylacridinium dissociation was still changing at 2.5×10^{-4} M *d*-tubocurarine since the K_D for *d*-tubocurarine in the ternary complex is 2×10^{-7} M. Have you measured the concentration dependence of *d*-tubocurarine-induced dissociation?

G. Mooser:

The dissociation constant for *d*-tubocurarine binding to the peripheral site on the free enzyme is about 8×10^{-7} M, but in the stopped flow experiments we are dealing with the formation of the *d*-tubocurarine—enzyme—*N*-MAC ternary complex where the dissociation constant is about 5×10^{-6} M. In the regression analysis the determination of these constants is not as accurate as those for the binary complexes and may be off by a factor of 2 or so. However, in this range it might be expected that the *d*-tubocurarine displacement of *N*-MAC would reach its maximum effects at 2.5×10^{-4} M, but not necessarily at 5×10^{-5} M. Another possibility suggested to me by Dr. Rosenberry is *d*-tubocurarine may form an initial weak complex with the enzyme which is subsequently stabilized in a slower step. We, of course, cannot distinguish these possibilities without examining the effects of *d*-tubocurarine over a wider range of concentrations.

SAŽETAK

Interakcija mjesta za vezivanje liganada na kolinesterazu

G. Mooser i D. S. Sigman

Istražena su svojstva acetilkolinesteraze vrste *Electrophorus electricus* s obzirom na vezivanje liganada u ravnotežnomu i u prijelaznim stanjima uporabom reversibilnih fluorescentnih spojeva, *N*-metilakridinija (*N*-MAC) i bis(3-amino-piridinijum)-1,10-dekana (DAP). *N*-MAC se veže u aktivnom mjestu, a DAP, analog deka-tonija, povezuje poput mosta aktivno mjesto i periferno nekatalitičko mjesto. Tim spojevima praćeno je vezivanje liganada, koji reagiraju s jednim ili s oba ova mjesta, na osnovi zamjene. Primjenom nelinearne regresije u pokusima zamjene fluorescentnih spojeva, utvrđeno je da se *d*-tubokurarin vezuje za mjesto udaljeno od katalitičkoga. Pri fiziološkoj ionskoj jakosti medija, *d*-tubokurarin se veže isključivo na periferno nekatalitičko mjesto a uz nisku ionsku jakost medija veže se na oba, i na periferno i na katalitičko mjesto. Uz obje ionske jakosti medija nađeno je, da je periferno nekatalitičko mjesto vezivanja ono isto, koje reagira sa sekundarnom kationskom funkcijom bis-kvarternih amonijevih spojeva, kao što su dekame-tonij i DAP. Kad je periferno mjesto zauzeto *d*-tubokurarinom, afinitet enzima za vezivanje liganada u aktivnom mjestu je smanjen. Pozitivna korelacija između veli-

čine liganada za aktivno mjesto i stupnja inducirane destabilizacije *d*-tubokurarinom, upućuje na mogućnost djelovanja nekih steričkih faktora. Reakcije vezivanja galamina i liganada u aktivno mjesto međusobno se isključuju. Spektrofotometrijskom metodom zaustavljenog toka istraženo je, da li je to posljedica vezivanja galamina u samom aktivnom mjestu ili se galamin veže na periferno nekatalitičko mjesto, što uzrokuje konformacijske promjene u samom aktivnom mjestu. Postojanje jednoga prijelaznog ternarnog kompleksa: galamin-enzim-N-MAC, upućuje na to, da je vjerojatnije vezivanje galamina na periferno mjesto.

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