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EVIDENCE THAT EEL ACETYLCHOLINESTERASE IS NOT AN INTEGRAL MEMBRANE PROTEIN

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Detergent binding studies indicated that the neural enzyme, acetylcholinesterase, did not exhibit the properties of an integral membrane protein. The 11S form was isolated by affinity chromatography from a tryptic digest and the 14S and 18S forms in like manner from an undigested preparation. Studies were performed with [^{3}H]TX-100 to determine the extent of binding by these forms and with catalase and human low density lipoprotein as reference proteins. All forms of the enzyme bound less than 0.04 mg TX-100/mg protein which is only slightly higher than binding by catalase and about 25 fold lower than the binding exhibited by low density lipoprotein.

1. Introduction

Recently, much of the research on acetylcholinesterase (E.C. 3.1.1.7) has centered upon the means by which this enzyme is attached to the neural or possibly basement membrane. In general, the problem appears to involve relative contributions between lipophilic and electrostatic or hydrogen bonding. Thus, the work of Brodbeck and coworkers [1,2] clearly shows the importance of TX-100 labile bonds (presumably lipophilic bonding sites) in the integration of acetylcholinesterase (AChE) into human erythrocyte membrane. Correspondingly, for eel and torpedo enzyme, the investigations of Massoulié and colleagues [3] as well as data from the laboratories of Rosenberry [4], Taylor [5], Dudai and Silman [6] and the work of Johnson et al. [7] present compelling evidence for the involvement of a salt sensitive binding mechanism involving a 500 nm collagen-like tail. However, the orientation and membrane location of AChE cannot be fully described without knowing the degree of lipophilicity possessed by the enzyme. If the enzyme has a high degree of lipophilicity, which can be detected by detergent binding studies [8], then penetration deep into the lipid bilayer is energetically feasible. A low degree of lipophilicity would preclude this arrangement. We present here studies on the TX-100 binding (lipophilicity) characteristics of several AChE sizeozymes [9].

2. Materials and methods

2.1. Chemicals

Electrophoretically and centrifugally pure 11S acetylcholinesterase (AChE) was isolated from a trypsin (E.C. 3.4.4.4, Worthington) digest of homogenized electric eel tissue (*E. electricus*, Paramount Research Supply Co., Ardsley, N.Y.) by affinity chromatography essentially according to Dudai et al. [10] followed by chromatography on hydroxylapatite [11]. 18S enzyme (a mixture of 18S and 14S enzymes) was prepared similarly by omitting the trypsin step. Human low density lipoprotein (LDL) was a gift of Dr. James Osborne. Catalase (E.C. 1.11.1.6) was obtained from Sigma. Hydroxylapatite was a Bio-Rad product; Aquasol was purchased from New England Nuclear, [^{3}H] TX-100 was purchased from Rohm and Haas and New England Nuclear. Several different stock preparations of different specific activity were used. Bovine albumin was a Pentex product, and Fluram was obtained from Roche

Diagnostics. All other chemicals were reagent grade or better.

2.2. Density gradient centrifugation

Five ml linear density gradients were constructed from 5% and 20% sucrose solutions containing 0.01 M sodium phosphate (pH 7.5) and 0.05% [^{3}H] TX-100 [12]. Operationally, 0.1 ml samples of protein (~ 2–3 mg/ml) in 0.01 M sodium phosphate (pH 7.5) containing 1% unlabeled TX-100 were layered onto the chilled gradients and centrifuged in a Beckman-Spinco L2-75B centrifuge using a SW50.1 rotor at 32000 rpm for 16 h at 4°C. The gradients were collected in 0.15 ml fractions and assayed for AChE activity using the Ellman assay [13]. One unit of AChE activity is defined as the change in absorbance at 412 nm/min. Catalase was assayed for enzymatic activity [14] and one unit of activity is defined as the change in absorbance at 240 nm/min. LDL was assayed for protein by reacting with the Fluram reagent using bovine albumin as standard [15]. 0.05 ml aliquots of the collected density gradient fractions were added to 10 ml aliquots of Aquasol and counted for radioactivity in a Packard TRI-CARB, model 3375, scintillation counter.

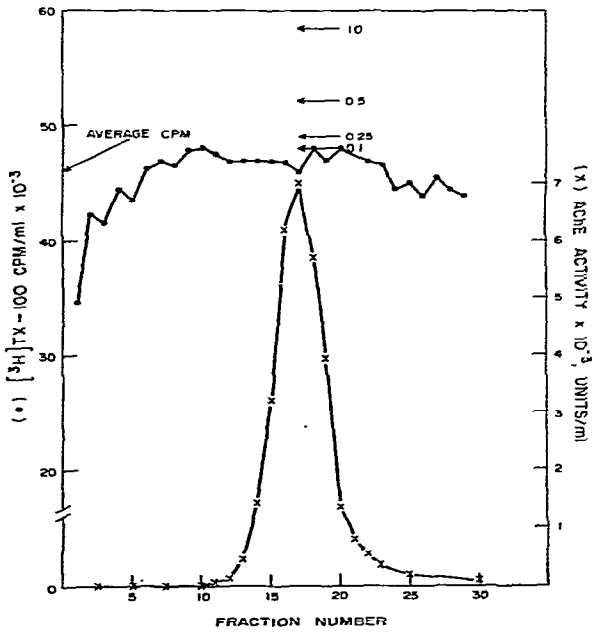

Fig. 1. Analysis of the binding of [^{3}H] TX-100 to 11S acetylcholinesterase using the sucrose density gradient technique of Clark [12]. There are approximately 0.2 mg/ml of enzyme in the peak tube. Other details are in the text.

2.3. Column chromatography

A column of hydroxylapatite (1.5 X 4 cm) was equilibrated with a minimum of 100 ml of 0.01 M sodium phosphate (pH 7.5). Protein samples were made up in and dialyzed overnight against this solvent, applied to the column, and then washed with the above buffer containing 0.05% unlabeled TX-100. The column was then washed with 0.01 M sodium phosphate (pH 7.5) containing 0.05% [^{3}H] TX-100. After this wash, protein was displaced from the column by washing with 0.3 M sodium phosphate (pH 7.5) containing 0.05% [^{3}H] TX-100. 0.2 ml aliquots of the collected fractions were added to 10 ml of Aquasol and counted for radioactivity. Protein peak tubes were counted in triplicate. Fluram determinations were performed for all proteins; in addition, AChE and catalase were estimated by enzyme activity.

3. Results and discussion

3.1. Sedimentation analysis

Fig. 1 shows the distribution of the radioactivity and AChE activity in a sucrose density gradient detergent binding experiment modeled after Clark [12]. Note that there is no discernable peak of radioactivity which is coincident with the enzyme activity peak. The horizontal arrows indicate the level of radioactivity which would have been attained had the peak tube of activity bound 1.0, 0.5, 0.25 mg, or 0.1 mg of TX-100 per mg of enzyme. Considering the fluctuation of radioactive counts within the plateau area (where no enzyme is present), this experiment suggests that if 11S AChE does bind detergent, a possible upper limit of binding is of the order of 0.1 mg detergent per mg protein. Similar results were obtained when catalase, a cytoplasmic protein and not expected to be lipophilic (12), was subjected to the same test. With LDL, we found approximately 0.9 ± 0.2 mg of detergent bound

per mg of LDL (determined in triplicate). This level of binding is to be compared with 0.9 mg/mg reported by Clark [12]. As fig. 1 shows, the sucrose density gradient binding technique did not possess sufficient sensitivity in our hands to accurately determine the actual lower binding limits for AChE due to the limited amount of protein (~100–300 μg) we could layer on the gradient and the high fluctuation in counts throughout the gradient. Therefore, we turned to the chromatographic method described below for resolution of this problem.

3.2. Chromatographic analysis

Figs. 2A, B, and C show the results of binding experiments conducted on hydroxylapatite columns at room temperature with 11S AChE and catalase and LDL as test proteins. The horizontal arrows in fig. 2A indicate the levels of radioactivity which would have been attained had the peak tube of 11S AChE bound 0.1, 0.05 or 0.025 mg of TX-100 per mg of enzyme. The experimental data indicate that if 11S AChE binds TX-100, the level of binding is at 0.025 mg/mg or below (~15 moles TX-100/mole enzyme). The finer discrimination achieved with the chromatographic technique versus the sedimentation technique (fig. 1) is apparent. This improvement is due to the larger amount of protein employed (~2 mg) and its subsequent elution in a relatively small volume. 11S AChE behaves more like catalase, fig. 2B, a cytoplasmic protein which gives no evidence of binding than it does LDL, fig. 2C, for which the binding is patent and at a level of 1.1 ± 0.2 mg/mg. This latter figure for LDL is in good agreement with literature values (12) and with the value determined by the sucrose gradient technique as reported above. For all these proteins, identical degrees of binding were found on repeating these experiments at 5°C.

Under our preparation conditions, 11S enzyme is

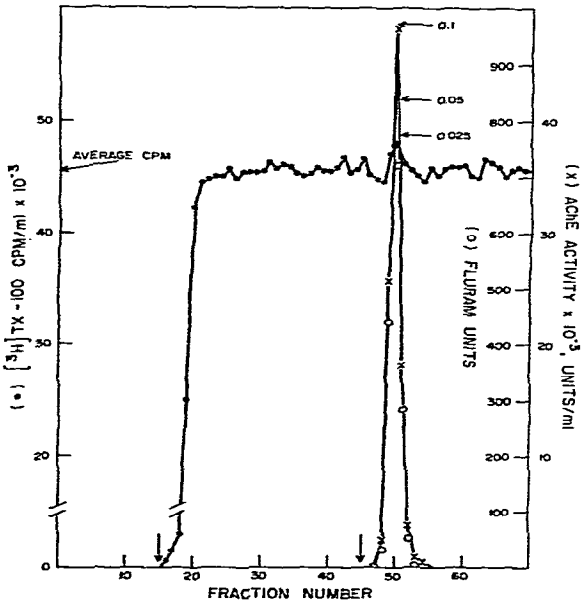

Fig. 2A. Analysis of the binding of [^{3}H]TX-100 to 11S acetylcholinesterase using hydroxylapatite chromatography. The vertical arrows indicate, respectively, introduction of 0.01 M phosphate buffer (pH 7.5) containing 0.05% [^{3}H]TX-100 and 0.3 M phosphate buffer (pH 7.5) containing 0.05% [^{3}H]TX-100. The peak tube contains approximately 1.4 mg/ml of enzyme. Fraction size: ~1.5 ml. Other details are in the text.

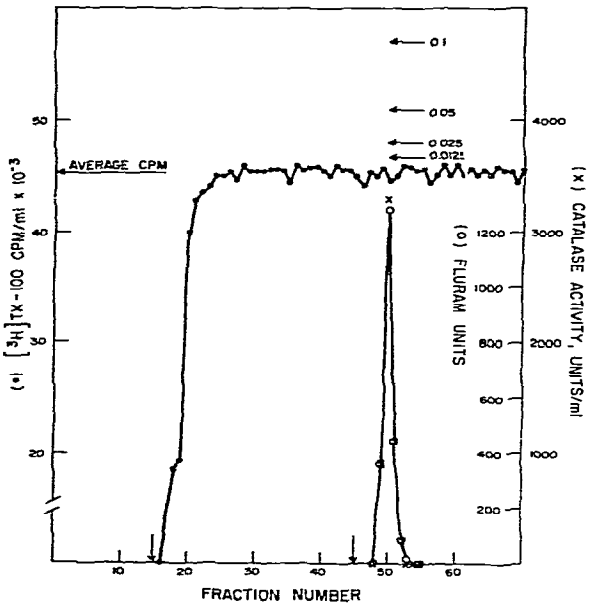

Fig. 2B. Analysis of the binding of [^{3}H]TX-100 to catalase using hydroxylapatite chromatography. The vertical arrows indicate, respectively, introduction of 0.01 M phosphate buffer (pH 7.5) containing 0.05% [^{3}H]TX-100 and 0.3 M phosphate buffer (pH 7.5) containing 0.05% [^{3}H]TX-100. There are approximately 1.5 mg/ml of catalase in the peak tube. Fraction size: ~1.5 ml. Other details are in the text.

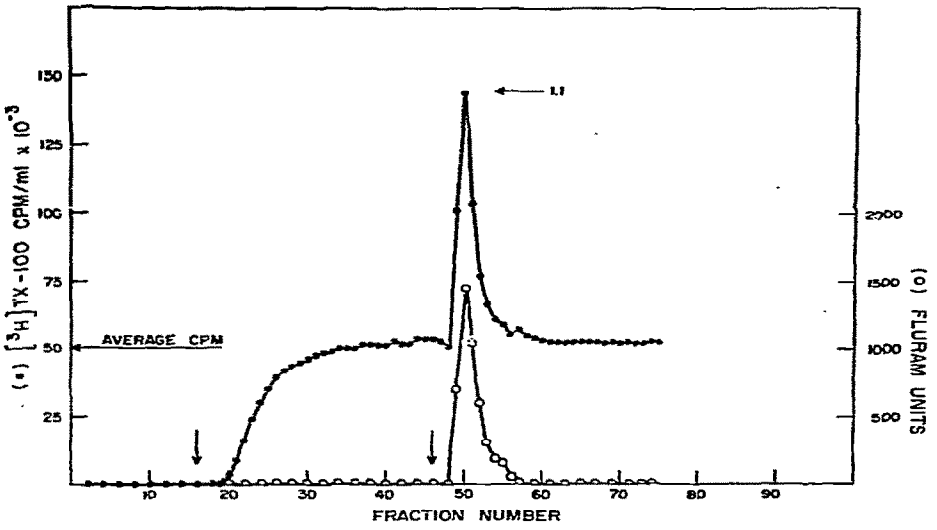

Fig. 2C. Analysis of the binding of [^{3}H] TX-100 to human low density lipoprotein using hydroxylapatite chromatography. The vertical arrows indicate, respectively, introduction of 0.01 M phosphate buffer (pH 7.5) containing 0.05% [^{3}H] TX-100 and 0.3 M phosphate buffer (pH 7.5) containing 0.05% [^{3}H] TX-100. There are approximately 1.9 mg/ml of LDL in the peak tube. Fraction size: ~1.5 ml. Other details are in the text.

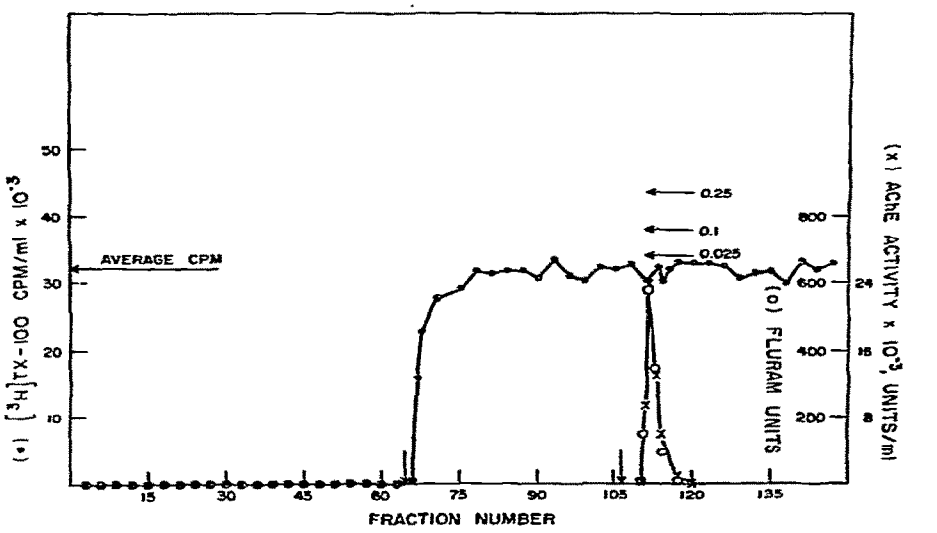

Fig. 3. Analysis of the binding of [^{3}H] TX-100 to 18S acetylcholinesterase using hydroxyiapatite chromatography. The vertical arrows indicate, respectively, introduction of 0.01 M phosphate buffer (pH 7.5) containing 0.05% [^{3}H] TX-100 and 0.3 M phosphate buffer (pH 7.5) containing 0.05% [^{3}H] TX-100. There are approximately 0.8 mg/ml of enzyme in the peak tube. Fraction size: ~0.8 ml. Other details are in the text.

proteolytically derived from 18S AChE, the form in which the enzyme most likely exists *in situ*. 18S enzyme is constructed of three tetramers grouped around one end of a tail estimated to be about 500 nm in length [5], whereas the 14S form has only two tetramers attached to the tail. The tail has been recently suggested to be of a collagen-like nature [4–7] and has also been reported to be the major distinction between the 11S and higher molecular weight forms [4–7]. Since the 11S tetramer does not exhibit the detergent binding characteristics of known integral membrane proteins, the possibility that the 18S enzyme *in situ* might be an integral membrane protein hinges on the possible lipophilic nature of the tail. Fig. 3 shows the results of a chromatographic binding experiment on a preparation of 18S AChE designed to test this alternative. Binding in this case is also very low, being about 0.025 to 0.034 mg TX-100/mg protein (42 to 57 moles TX-100/mole 18S). This range was calculated using binding values of 0.025 or zero mg TX-100 per mg of 11S in this preparation (sucrose density gradient analysis performed on the day of the experiment gave 12% 11S enzyme and 88% of a mixture of 18S + 14S enzymes). If the 18S tail contained a distribution of hydrophobic residues such that it bound at least the equivalent of one micelle of TX-100 (as many other membrane proteins appear to do [16] and see table 1) and assuming the remainder of 18S protein to bind no TX-100, the level of binding we should have observed was of the order of 0.06 to 0.07 mg/mg of 18S. The observed binding is much less, being comparable on a molar basis, to the major RBC glycoproteins (table 1). The results presented above show that various forms of the neural enzyme, AChE, as isolated from the electric eel bind small amounts of TX-100 (when expressed as mg TX-100 bound per mg protein) and in this respect do not behave operationally as most integral membrane proteins do ([12] and table 1). However, when expressed on a molar basis, the similarity of amounts of TX-100 bound by the ma-

Table 1
Binding of TX-100 to lipophilic and lipophobic proteins

Protein	Molecular weight	mg TX-100 bound / mg protein	Moles TX-100 bound / Mole protein	Ref.
A. Cytochrome b5	16,200	4.0	100	[16]
Minor RBC glycoprotein	175,000	0.77	207	[12]
Major RBC glycoprotein	28,000	1.12	48	[12]
Glycoproteins	1,100,000	0.21	355	[19]
SF virus membrane	150,000	0.54	125	[19]
Opsin	75,000	0.70	81	[12]
Cytochrome oxidase	308,000	0.3	142	[19]
Cholinergic receptor	470,000	0.3	216	[19]
Rhodopsin	39,100	1.64	99	[21]
Rhodopsin	39,100	1.10	66	[12]
B. Bovine serum albumin	68,000	0.025	2.6	[12]
Human serum albumin	68,000	0.07	7.3	[20]
Aldolase	149,100	<0.02	<4.6	[12]
Chymotrypsinogen	23,240	<0.03	<1.1	[20]
Cytochrome C	13,370	<0.02	<0.4	[12]
G3P Dehydrogenase	136,000	<0.02	<4.2	[12]
γ-Globulin	153,100	<0.05	<12.6	[20]
Hemoglobin	64,000	<0.01	<1.0	[12]
Lysozyme	17,200	<0.02	<0.53	[12]
Ovalbumin	43,500	<0.03	<2.0	[20]
Ovalbumin	43,500	<0.01	<0.67	[12]
Human transferrin	74,000	<0.05	<5.7	[20]

jor RBC glycoprotein (itself an integral membrane protein [12]) and the 18S acetylcholinesterase might lead to the unwarranted speculation that 18S enzyme is an integral membrane protein. Another potentially useful means of measuring the relative lipophilicities of TX-100 binding proteins would be to compare the percentage of protein surface area occupied by the bound detergent. This calculation is made possible by the fact that TX-100 binding does not denature proteins and hence result in a major conformational change. For the sake of convenience, the proteins have been treated as spheres and thus the calculated surface areas only serve as *crude* approximations to the true surface area. In these calculations, the contact area per detergent molecule of average MW 650 was taken as 70 $Å^2$ [17]. These calculations showed for the proteins of Section A that bound TX-100 "occupied" 40% (cytochrome oxidase) to 200% (cytochrome b5) of the theoretical surface area. The very high value for cytochrome b5 presumably reflects both micellar binding and the fact that TX-100 binds only to a small portion of the protein [16]. The cytoplasmic and circulating proteins (for which a spherical model more closely approximates the true shape) have only 0.7% (hemoglobin) to 6% (human serum albumin) of their surface area occupied by bound TX-100. For 11S and 18S acetylcholinesterase, and *assuming* the data in figs. 2A and 3 represent *maximal* detergent binding, we calculate 3 and 5% of their surface area could be "occupied" by bound TX-100. Consequently, the surface of AChE appears to be devoid of large, accessible hydrophobic regions. Hence, deployment of the bulk of the AChE molecule deep into a lipid bilayer would seem to be difficult. The recent findings of Watkins et al. [18] that sphingomyelin but not phosphatidylcholine binds to "tailed" AChE in a salt dependent manner may indicate that a highly specialized geometry of the AChE binding vehicle is required. Our data suggest that binding of eel AChE to the neural membrane or synaptic structural components is not effected by a large scale hydrophobic lipid-protein interaction such as those demonstrated for integral membrane proteins [8, 12, 16].

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