HOMOLOGOUS CHOLINERGIC FLUORESCENT PROBES. SYNTHESIS AND FLUORESCENCE PROPERTIES

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1. Introduction

The advantages of using specifically designed fluorescent molecules to probe ligand binding sites on proteins, have been recognised [1]. Cholinesterases [2–4], acetylcholine receptor rich fragments from *Torpedo marmorata* [5,6] and an acetylcholine binding lipoprotein [7] have been studied using fluorescent trialkylammonium ligands. In these investigations, the probes incorporated an ethane [7] or propane bridge [3,5] between the fluorescent dansyl group and the trimethylammonium moiety. During the course of studies of cholinergic ligand binding to mammalian cell plasma membranes, we became aware of the need for developing cholinergic fluorescent probes with increased lipid solubility and higher binding affinities. The lengthening of the spacer alkyl chain appeared an attractive modification. This would further allow investigations of the hydrophobic areas postulated in the vicinity of the active site in cholinesterases [8,9]. We report here the synthesis of three homologous cholinergic fluorescent probes (II, V and X, fig.1) and describe results of binding studies with cholinesterases.

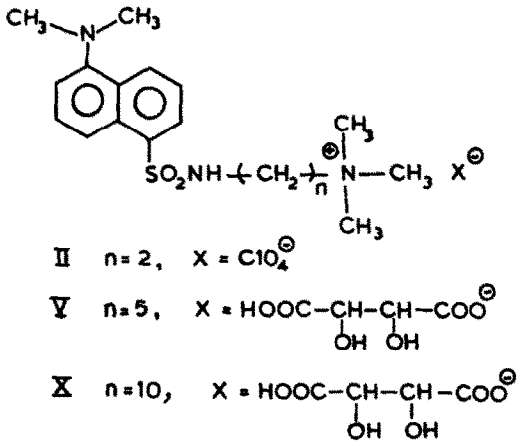

Fig.1. Structures of fluorescent probes.

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2. Experimental

2.1. *Synthetic procedures*

Dansyl chloride was prepared as in [10].

2.1.1. 1-(5-Dimethylaminonaphthalene-1-sulfonamido)ethane-2-trimethylammonium perchlorate (II)

This was synthesised from dansyl chloride and *N,N*-dimethylethylene diamine [11] as in [7]: yield 70–80%; m.p. 181–183°C.

2.1.2. 1-(5-Dimethylaminonaphthalene-1-sulfonamido)pentane-5-trimethylammonium tartarate (V)

Cadaverine obtained from Sigma Chemical Co. was converted to dansyl cadaverine as in [12]. Exhaustive methylation of dansyl cadaverine was effected by the procedure in [13]. 300 mg (2 equiv.) pentamethyl

piperidine and 2 ml cold methyl iodide were successively added to a cold, stirred solution of 350 mg dansyl cadaverine in 20 ml ethyl acetate. Careful filtration of the precipitated pentamethyl piperidine hydriodide and evaporation of the solvent under reduced pressure yielded the iodide salt of V (65–75%). The iodide salt was dissolved in 5 ml methanol and 2–3 equiv. of tartaric acid was added. The tartarate salt of V then crystallised; m.p. 137–138°C.

2.1.3. 1-(5-Dimethylaminonaphthalene-1-sulfonamido) decane-10-trimethylammonium tartarate (X)

1,10 Dodecanedioic acid (Sigma) was converted to 1,10-decamethylene diamine by the Schmidt reaction [14]. A solution of 800 mg dansyl chloride in 50 ml dry ether was added to 2 g diamine in 200 ml dry ether at room temperature, in a nitrogen atmosphere, over 3 h. After 1 h more stirring, the ether was filtered and the residual fine solid taken up in 1 M sodium bicarbonate and methylene chloride. The organic layer was washed with 2 X 50 ml of 1 M bicarbonate, water, dried over sodium sulfate. The ether layer was similarly treated, both solvent layers combined and evaporated under reduced pressure to give a green fluorescent oil which was chromatographed on a silica gel column. Elution with a 10 : 1 mixture of chloroform and methanol gave 470 mg required alkylamine. Exhaustive methylation of the alkylamine by the method described for V gave the iodide salt in 80% yield. Conversion to the tartarate was carried out as for V; m.p. 140–141°C.

The three probes were chromatographically homogenous on silica gel and gave satisfactory 100 MHz and 270 MHz ^{1}H NMR spectra.

2.2. *Fluorescence measurements*

Fluorescence experiments were performed on a Perkin Elmer Model 203 spectrofluorimeter. The probe excitation wavelength was 340 nm while protein tryptophan residues were excited at 290 nm. Matched 1 cm cuvettes were used. All solutions were made in 10 mM Tris.HCl buffer, pH 8.

Horse serum cholinesterase type IV, 15.7 units/mg solid; electric eel acetylcholinesterase type VI, 225 units/mg solid; bovine erythrocyte acetylcholinesterase, 2.6 units/mg solid; bovine serum albumin, α-chymotrypsin and lysozyme were obtained from Sigma Chemical Co. One unit cholinesterase hydrolyses 1 μM acetylcholine/min at pH 8.0; 37°C.

3. Results and discussion

The changes in the fluorescence spectra of II, V and X on binding to horse serum cholinesterase (HSChE) are shown in fig.2. Interaction with the enzyme is accompanied by an enhancement of the emission intensity and a blue shift of the fluorescence maximum. The magnitude of the spectral changes follows the order X > V > II, suggesting an increase in binding with the lengthening of the alkyl chain. Figure 3 shows the fluorescence spectra of V in the presence of the proteins HSChE, electric eel acetylcholinesterase (Type VI, Sigma), erythrocyte acetylcholinesterase, lysozyme and bovine serum albumin (BSA). Table 1 summarises the results of fluorescence experiments with II, V and X in the presence of various proteins. It is interesting that the emission properties of all three probes are altered in the presence of HSChE while electric eel and bovine erythrocyte acetylcholinesterases do not cause large changes. A similar observation was reported for a cholinergic probe [2]. The probes II, V and X have been shown to be inhibitors of HSChE and membrane bound acetylcholinesterase. For HSChE, the extent of inhibition follows the order X > V > II. Further, covalent blocking of the enzyme site by eserine leads to diminution in the fluorescence enhancement for X. The data in table 1 shows that while the fluorescence of V is unaffected in the presence of BSA, X shows an intensity enhancement and blue shift, suggestive of probe binding. This could conceivably arise as a consequence of the interaction of X with sites that have an affinity for trialkylammonium ligands. It is interesting to note that BSA has been shown to bind phosphatidyl choline molecules [15]. Further experiments are however needed to establish the specificity of the binding of X to BSA.

Fluorescence titrations varying probe and protein concentrations [16] were carried out for the binding of II, V and X with HSChE. The double reciprocal plot of the fluorescence intensity as a function of protein concentration, yielded linear plots extrapolating to the same value of the fluorescence of the bound probe for II, V and X. Scatchard plots [16]

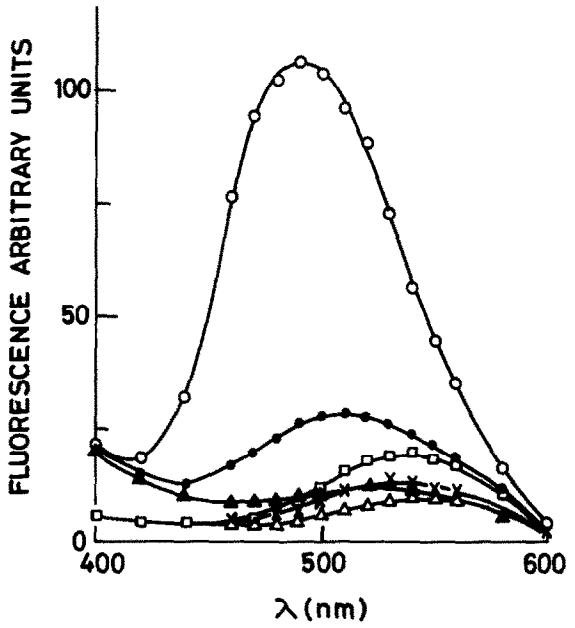

Fig.2. Uncorrected fluorescence spectra of probes II, V and X in the presence of horse serum cholinesterase. Excitation 340 nm. Probe conc. 2 μM. Protein conc. 200 μg/ml. (△) II in buffer. (▲) II + protein. (□) V in buffer. (●) V + protein. (X) X in buffer. (○) X + protein.

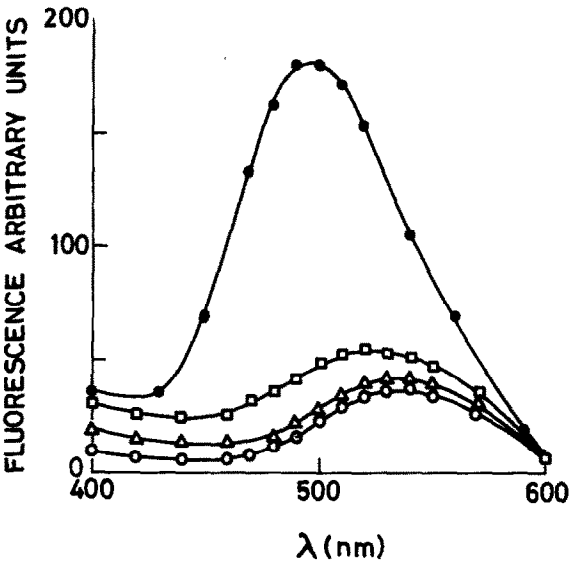

Fig.3. Uncorrected fluorescence spectra of V in the presence of proteins. Excitation 340 nm. Probe conc. 2 μM. Protein conc. 200 μg/ml. (○) V in buffer. (●) V + horse serum cholinesterase. (△) V + BSA. (□) V + electric eel acetylcholinesterase. The spectra of V + erythrocyte acetylcholinesterase and V + lysozyme coincide with the spectrum of V in buffer.

Table 1
Fluorescence intensities for probes in the presence of proteins

Protein	II		V		X	
	λ_{max}	Relative intensity	λ_{max}	Relative intensity	λ_{max}	Relative intensity
Horse serum cholinesterase	525	1.3	510	1.47	490	8.15
Electric eel acetylcholinesterase	540	1.0	530	1.20	530	1.30
Bovine erythrocyte acetylcholinesterase	540	1.0	540	1.00	530	1.30
BSA	540	1.1	530	1.15	490	10.00
α-Chymotrypsin	540	1.0	540	1.00	535	1.30
Lysozyme	540	1.0	540	1.00	540	1.25

Probe conc. 2 μM; protein conc. 200 μg/ml

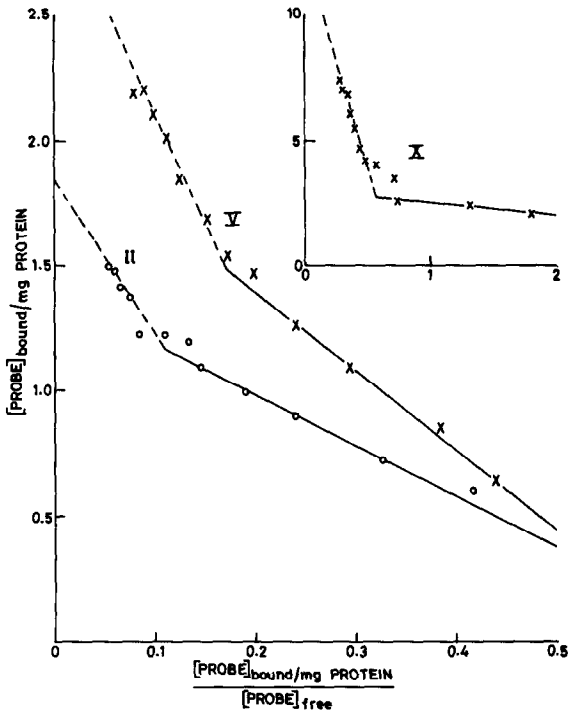

Fig.4. Scatchard plot for the binding of II, V and X to horse serum cholinesterase. Excitation 340 nm. Emission 500 nm. Protein conc. 0–28 μM. *X* axis units and *Y* axis units are litre. g^{-1} and mol. g^{-1}, respectively.

representing the binding data are shown in fig.4. The plots for all three probes show deviations from linearity over the range of probe concentrations studied (0–28 μM). These observations suggest binding site heterogeneity and the data points to the existence of at least two categories of binding sites for X. For II and V binding to the weaker site is less evident over the range of probe concentrations used. It may be noted that the scales for the Scatchard plots for II and V (fig.4) are enlarged relative to X. This is necessitated by the much larger fluorescence changes for X on interaction with HSChE. As a consequence the curvature in the plots for II and V, in fig.4 is magnified. Table 2 lists the dissociation constants (K_D) and the binding stoichiometries *(n)* for the interaction of the probes with HSChE, derived from fig.4. The results suggest that X interacts with two sites of widely differing affinities, while II and V probe sites of similar affinities ($K_D \sim 10^{-6}$ M), over this concentration range. A recent analysis of the binding of fluorescent probes to proteins and membranes has pointed out the errors arising from the linear extrapolation of double reciprocal plots of fluorescence data [17]. However, for the purposes of comparing the interaction of II, V and X with HSChE, the conventional Scatchard treatment [16] is adequate even though the absolute values of *n* and to a lesser extent, K_D, may be subject to error.

Table 2
Dissociation constants (K_D) and binding stoichiometries *(n)* for probes binding to horse serum cholinesterase

Probe	n_1 (μmol/mg)	K_{D_1} (M)[1]	n_2 (μmol/mg)	K_{D_2} (M)[2]
II	1.39	2.03×10^{-6}	1.84	6.17×10^{-6}
V	2.03	3.10×10^{-6}	2.96	8.71×10^{-6}
X	3.00	4.97×10^{-7}	12.00	1.67×10^{-5}

Cholinesterases are known to possess secondary binding sites in addition to the main catalytic anionic site [18]. Evidence also exists for the presence of hydrophobic areas in the vicinity of the active site [8,9]. While all three probes presumably interact with the catalytic anionic site, X also shows evidence for binding to other sites on the protein. While the primary binding force is the electrostatic interaction of the trimethylammonium group with anionic residues on the enzyme, additional stabilising interactions involving the non-polar alkyl chain and naphthalene ring with hydrophobic areas on the protein are also possible. The fluorescence data presented here support the idea that X may be structurally more capable of probing these areas on the enzyme surface, than II or V. ^{1}H NMR studies at 100 MHz (results not shown) have established a significantly larger increase in the linewidth of the $N(CH_3)_2$ protons relative to the $+N(CH_3)_3$ protons for the probes in the presence of HSChE. This enhancement in the spin–spin relaxation rate provides clear evidence for immobilisation of the $N(CH_3)_2$ group of the probes on binding to the protein. Details of the NMR studies, enzyme inhibition and competitive inhibitor binding studies will be discussed elsewhere.

Figure 5 shows the energy transfer spectra of X in the presence of HSChE. Addition of probe leads to a quenching of the protein tryptophan fluorescence while the emission intensity at 495 nm increases due to excitation energy transfer, arising from proximate

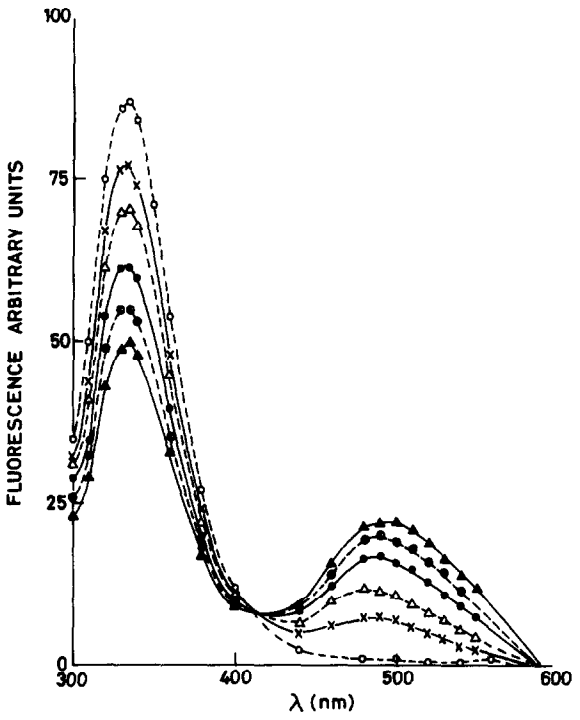

Fig.5. Uncorrected fluorescence spectra of the effect of X on horse serum cholinesterase tryptophan fluorescence. Excitation 285 nm. Protein conc. 100 μg/ml. Probe conc.: 0 μM (○), 1.96 μM (×), 3.85 μM (△), 7.41 μM (●), 10.71 μM (⊛) and 13.79 μM (▲).

tryptophan residues. Similar results for energy transfer from the binding protein to a cholinergic probe have been reported [2]. The results presented above demonstrate that increasing the length of the alkyl chain separating the dansyl group from the trimethylammonium group leads to enhanced binding to cholinesterases. The much greater fluorescence changes on binding obtained for X as compared to II and V, suggest that X may serve as a more sensitive probe of cholinergic ligand–protein interactions.

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