Int. J. Peptide Protein Res. 17, 1981, 170–180

FLUORESCENCE AND N.M.R. STUDIES OF THE BINDING OF CHOLINERGIC FLUORESCENT PROBES TO HORSE SERUM CHOLINESTERASE

REVATHI NARAYANAN and P. BALARAM

Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India

Received 11 February, accepted for publication 23 June 1980

The interaction of the cholinergic fluorescent probes, 1-(5-dimethylaminoaphthalene-1-sulfonamido) ethane-2-trimethylammonium perchlorate, 1-(5-dimethylaminonaphthalene-1-sulfonamido) pentane-5-trimethylammonium tartarate and 1-(5-dimethylaminonaphthalene-1-sulfonamido) decane-10- trimethylammonium tartarate with horse serum cholinesterase has been examined by fluorescence and n.m.r. methods. Fluorescence titrations show binding of the decane derivative to two sites on the protein whereas the lower homologs bind largely to one site. Active site inhibitors like carbamylcholine and decamethonium abolish binding of the decane derivative to the high affinity site. The inhibitors are largely without effect on the binding of the lower homologs. N.m.r. studies clearly establish immobilization of both ends of the molecule on binding in the case of the decane derivative, whereas in the lower homologs the dimethylamino group on the naphthalene ring is significantly more affected in the presence of enzyme. The probes are effective inhibitors of the enzyme with the decane derivative being two orders of magnitude more effective than its lower homologs. Based on the n.m.r., fluorescence and inhibition studies, a model for probe binding to the enzyme is advanced. It appears that the decane derivative binds with high affinity to the catalytic anionic site while the lower affinity site is assigned to a peripheral anionic site. The lower homologs probe only the peripheral site. A comparison of fluorescence, n.m.r. and inhibition studies with acetylcholinesterases from electric eel and bovine erythrocytes is presented.

Key words: cholinergic inhibitors; cholinergic probes; cholinesterase; fluorescence; fluorescent probes; nuclear magnetic resonance.

The concept of "active site direction" has been used to advantage in fluorescent probe studies of cholinesterases (Himel & Chan, 1976; Cohen & Changeux, 1973; Himel *et al.*, 1971). The existence of a main catalytic site, one or more peripheral sites and hydrophobic domains near the active site, has been postulated for serum cholinesterase (Kabachnik *et al.*, 1970) and for acetylcholinesterase (Rosenberry, 1975). These studies show that there are structural and topo-

* Abbreviations used: HSChE, horse serum cholinesterase; AChE, acetylcholinesterase; DM, decamethonium; HM, hexamethonium; CC, carbamylcholine; TC, d-tubocurarine. The compounds QII, QV and QX were abbreviated as II, V and X in the earlier report (Narayanan & Balaram, 1978).

0367–8377/81/010170–11 $02.00/0

graphical differences between the two classes of enzymes.

We have shown in an earlier study (Narayanan & Balaram, 1978) that the cholinergic probes, 1-(5-dimethylaminonaphthalene-1-sulfonamido) ethane-2-trimethylammonium perchlorate, QII, 1-(5-dimethylaminonaphthalene-1-sulfonamido) pentane-5 trimethylammonium tartarate, QV and 1-(5-dimethylaminonaphthalene-1-sulfonamido) decane-10-trimethylammonium tartarate, QX (Fig. 1A), bind to horse serum cholinesterase (HSChE) but not to many other proteins. Further, the binding affinities increase with increasing length of the spacer alkyl chain. In this study, the effects of cholinergic inhibitors on the binding of these probes to HSChE, are described. The data from fluorescence experiments has been correlated with the results of enzyme inhibition and n.m.r. studies. The information obtained from the three techniques has been discussed in terms of a possible model for probe binding to the enzyme.

MATERIALS AND METHODS

Horse serum cholinesterase Type IV-S (15.7 units/mg solid, 1 unit hydrolyses 1 μM acetylcholine per minute at pH 8, 37°), acetylcholinesterase Type VI from electric eel (225 units/mg solid), acetylcholinesterase Type I from bovine erythrocytes (2.6 units/mg solid), hexamethonium bromide, decamethonium bromide, carbamylcholine chloride, d-tubocurarine chloride, dithiobisnitrobenzoic acid (DTNB) and acetylthiocholine iodide were obtained from Sigma Chemical Company, USA. The synthetic procedures adopted for the fluorescent probes, QII, QV and QX have been reported earlier (Narayanan & Balaram, 1978).

Fluorescence experiments

All solutions were prepared in 10 mM Tris-HCl buffer, pH 8. Fluorescence measurements were made on a manual Perkin-Elmer Spectrofluorimeter (Model 203) and are uncorrected. Matched 1-cm cuvettes were used. The excitation wavelength is 340 nm. Titrations were performed using a constant probe concentration of 2 μM while protein concentrations were varied between 0 and 280 μg/ml. Probe concentrations were varied between 0 and 30 μM at a constant protein concentration of 200 μg/ml. Inhibitor concentrations were fixed at 200 μM. The binding parameters were calculated by the method described by Azzi (1974) and represented as Scatchard plots (Scatchard, 1949).

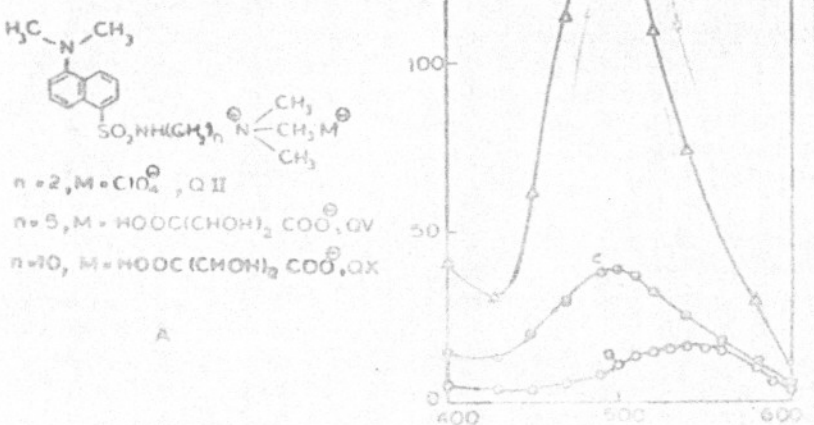


FIGURE 1

A, Structures of QII, QV and QX. B, Effect of decamethonium on the fluorescence of QX in the presence of HSChE. Probe, protein and decamethonium concentrations 2 μM, 200 μg/ml and 30 μM, and 280 μM respectively; for QX in buffer, (b) QX + HSChE, (c) QX + HSChE + decamethonium.

Kinetic measurements

Horse serum cholinesterase and acetylcholinesterase from electric eel and bovine erythrocytes were assayed essentially by the method of Ellman *et al.* (1961). In a typical run, the total reaction mixture was made up to 7.5 ml in 0.1 M phosphate (pH 8.0). For HSChE, the mixture contained 0.05 ml enzyme (500 μg/ml), 0.25 ml DTNB while substrate was varied between 0.025 and 0.15 ml. Blanks were adjusted to 100% transmission without substrate. The change in optical density on addition of substrate was followed at 412 nm for 5 min at 30-second intervals. The kinetics of AChE from bovine erythrocytes were studied under identical conditions. AChE from electrical eel was assayed using an enzyme stock of 8.33 μg/ml. The concentration of QX was varied between 0 and 0.08 μM for HSChE and 0 and 5 μM for AChE. QII and QV concentrations were varied between 0 and 10 μM. The data is represented by Lineweaver-Burke plots taking V, the velocity of enzyme hydrolysis, as ΔA, the change in adsorption per minute. The negative intercept of a plot of the slopes of the Lineweaver-Burke plots against appropriate probe concentrations gives the value of the inhibition constant, K_I.

Nuclear magnetic resonance (n.m.r.) measurements

N.m.r. experiments were performed on a Varian HA 100D instrument and a Bruker WH-270 Fourier Transform N.m.r. Spectrometer at the Bangalore N.m.r. Facility. All probe and protein solutions were made in D_2O. The pH of these solutions was adjusted between 7.8 and 8.2 with concentrated NaOD and DCl such that the volume changes were minimal The probe concentration was generally 1 mg/ml. HSChE concentration was varied between 0 and 4 mg/ml for QII and QV and between 0 and 0.25 mg/ml for QX. At 100 MHz, signal to noise ratios were improved using a Varian C-1024 time averaging computer. Scan times of 50 or 100 s were used for a sweep width of 100 Hz and satisfactory S/N ratios were obtained in 30–50 scans. At 270 MHz, sweep widths of 3000 Hz were used with 8K data points. Chemical shifts are expressed as δ (p.p.m.) downfield from 4, 4-dimethyl-4-silapentane-1-sulfonate. These values are obtained using a shift of 4.76 for the residual HDO resonance.

RESULTS

Fluorescence studies

In order to demonstrate that probe binding to HSChE involved the catalytic anionic site and/or the peripheral sites of the protein, the effects of the cholinergic inhibitors, decamethonium bromide (DM) and eserine, on the fluorescence of the QX-HSChE complex were studied. Binding of QX to HSChE results in a blue shift of about 50 nm in the emission maximum. This suggests binding of the probe to a hydrophobic region on the protein. Fig. 1B shows that bound QX fluorescence intensity diminishes on the addition of DM. However, there is no change in the position of the emission maximum. A decrease of 20% in bound QX intensity is also seen on the addition of as little as 8 μM eserine. This carbamate inhibitor affects enzyme activity by reacting covalently with residues at the active site (Main & Hastings, 1966). The diminution of bound probe fluorescence then arises from reduced probe binding at the active site. Decamethonium, however, interacts noncovalently with HSChE and AChE and shows mixed inhibition patterns with both enzymes (Kamaric 1975*a*, *b*). Therefore, the reduction in bound probe fluorescence, in the presence of DM, could be the result of direct displacement or alternatively, of structural changes in probe binding sites on interaction with the inhibitor. Cholinesterases are subject to allosteric control (Changeux, 1966) and the results obtained with DM and eserine reinforce the view that the probes bind to the catalytic anionic site or the peripheral anionic site or both.

Earlier studies (Narayanan & Balaram, 1978) have shown that the Scatchard plot for the binding of QX to HSChE is non-linear, suggesting the existence of two classes of binding sites ($K_D \sim 10^{-7}$ for Site 1 and $K_D \sim 10^{-5}$ for Site 2). The binding parameters are listed in Table 1. While binding site heterogeneity is clearly seen for QX, the deviations from linearity of the Scatchard plots for QII and QV are very much smaller. An attempt has been made to fit this data to obtain two sets of binding parameters. However, over this concentration range, the values of n and K_D obtained are very similar, suggesting that QII and QV effectively probe only a single class of binding

TABLE 1

Effect of cholinergic inhibitors on the binding parameters for the interaction of QV and QX with horse serum cholinesterase[a].

Probe	Inhibitor	n_1 (μmol g^{-1})	K_{D_1} (M)	n_2 (μmol g^{-1})	K_{D_2} (M)
QII	-	1.39	2.03×10^{-6}	1.84[b]	6.17×10^{-6}[b]
QV	-	1.99	3.06×10^{-6}	3.06[b]	9.27×10^{-6}[b]
QV	HM	1.35	2.25×10^{-6}		
QV	DM	1.60	2.29×10^{-6}		
QV	CC	1.50	1.76×10^{-6}		
QV	TC	1.25	1.79×10^{-6}		
QX	-	3.26	6.48×10^{-7}	11.00	1.29×10^{-6}
QX	HM	4.00	7.68×10^{-7}	11.00	1.10×10^{-6}
QX	DM	-	-	7.75	1.11×10^{-6}
QX	CC	-	-	6.00	1.10×10^{-6}
QX	TC	5.64	11.70×10^{-7}	16.00	1.12×10^{-6}

a Data obtained from Figs. 2 and 3. Units for 'n' in earlier publication (Narayanan & Balaram, 1978) should be μmol g^{-1}. The small differences that can be seen between the values of n and K_D reported earlier and in the present study are a reflection of the errors in the determination of binding parameters from different sets of experiments.

b n and K_D values obtained from the high probe concentration region of the Scatchard plot.

sites. The n and K_D values obtained at lower probe concentration are more likely to reflect the true binding parameters for this site. In order to further understand the nature of the probe binding sites, the effects of the cholinergic inhibitors, decamethonium (DM), hexamethonium (HM), carbamylcholine (CC) and d-tubocurarine (TC), on the interaction of the probes with HSChE were investigated.

Figs. 2 and 3 show the Scatchard plots for the binding of QV and QX to HSChE in presence of the four inhibitors. The value of F_m, the limiting fluorescence per μM of bound probe, remains unaltered in the presence of DM, HM and CC. This implies that the bound probe quantum yield is unaffected by inhibitor binding and that the microenvironments of the bound probe are unaltered in the presence of inhibitor. In the case of TC (figure not shown) there is a distinct lowering of F_m. This inhibitor is, however, susceptible to photodegradation and gives rise to fluorescent byproducts, which may lead to errors in the values of measured fluorescence intensity.

The binding stoichiometries (n) and dissociation constants (K_D) determined for QV and QX in the presence of the four inhibitors are listed in Table 1. The data for QV shows that, over the range of concentrations studied,

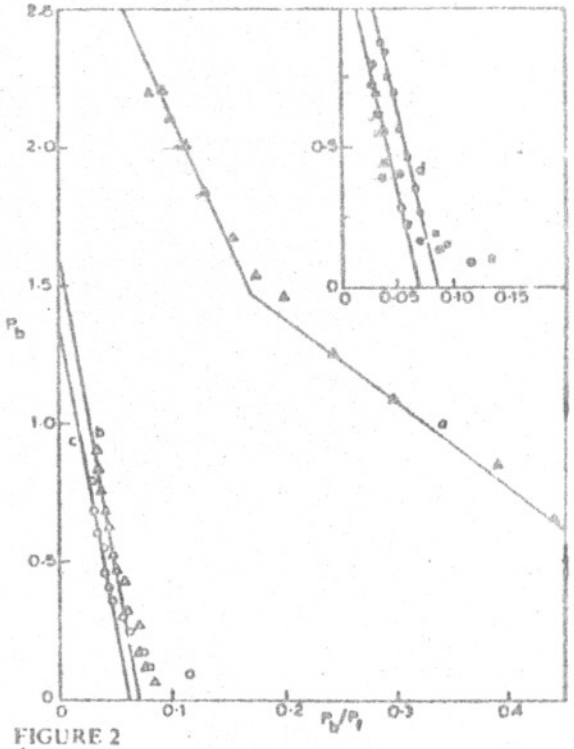


FIGURE 2

Scatchard plots for the interaction of QV with HSChE in the presence of cholinergic inhibitors. P_b and P_f are the concentrations of bound and free probe respectively. (a) Buffer. (b) Decamethonium. (c) Hexamethonium. (d) Carbamylcholine. (e) d-Tubocurarine.

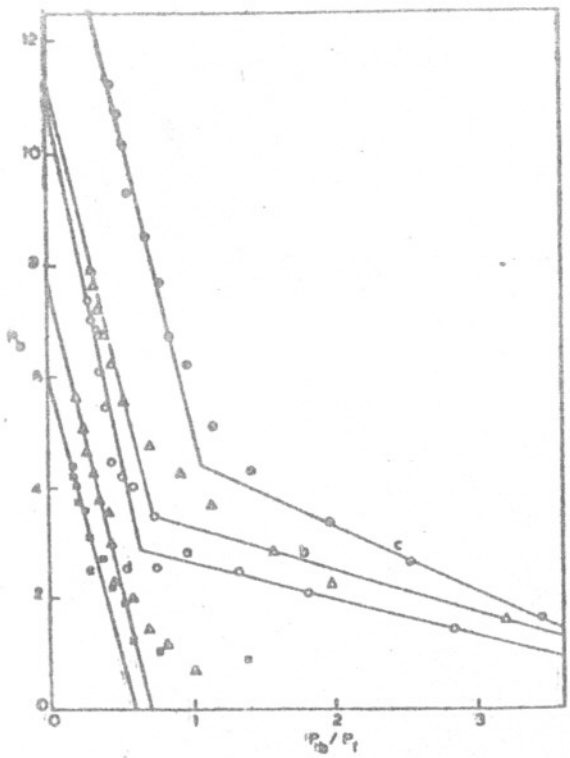


FIGURE 3

Scatchard plots for the interaction of QX with HSChE in the presence of inhibitors. P_b and P_f are the concentrations of bound and free probe, respectively. (a) Buffer. (b) Hexamethonium. (c) d-Tubocurarine. (d) Decamethonium. (e) Carbamylcholine.

the four inhibitors seem to have similar effects on the fluorescence of the QV-HSChE complex. This result must be contrasted with the binding of QX to HSChE in the presence of the inhibitors. Fig. 3 shows that while DM and CC cause a marked reduction in the non-linearity of the QX binding plot, two sets of binding parameters may still be obtained in the presence of HM and TC.

Inhibition studies

Fig. 4A shows the Lineweaver-Burke plot for the inhibition of HSChE by QII. Similar results were obtained for QV. Both these probes display the characteristics of mixed inhibition (Ayling, 1973). Fig. 4B shows the corresponding plot for QX, which suggests competitive binding to HSChE. Inhibition data for QII and QV (dashed lines) are also shown for comparison. It can be seen that the order of inhibition is QX (0.08 μM) > QV (4.3 μM) > QII (4.3 μM). It should be noted that QX is almost two orders of magnitude more effective in inhibiting HSChE. Fig. 5 shows the plots of S/V versus inhibitor concentration, for the inhibition of HSChE by the three probes. The K_i values determined from Fig. 5 together with the values obtained for the inhibition of electric eel AChE and bovine erythrocyte AChE by QX are summarized in Table 2.

QX shows mixed inhibition of the two acetylcholinesterases. The results show that QX is a less effective inhibitor of these two enzymes, as compared to HSChE. However, the K_i values are still indicative of fairly strong inhibition of these enzymes by QX. It was earlier shown that there are no major changes in the fluorescence parameters of QX in the

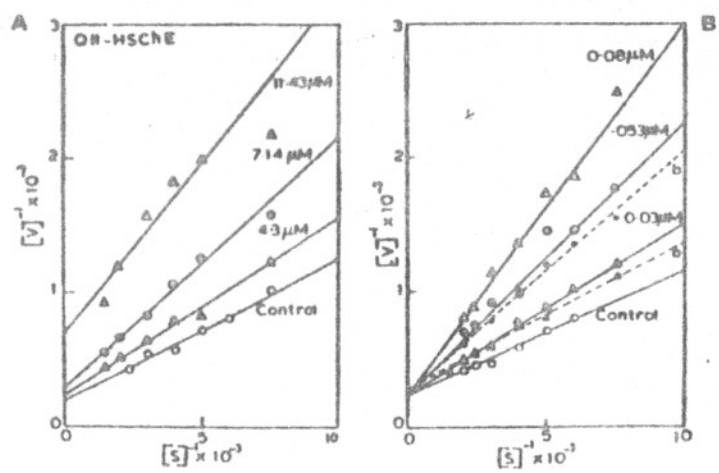


FIGURE 4

A, Lineweaver-Burke plots of the inhibition of HSChE by QII. V is the rate of change in OD. S is the substrate concentration in mol liter^{-1}. B, Lineweaver-Burke plots of the inhibition of HSChE by QX (solid lines). The broken lines represent (a) inhibition by QII (4.3 μM; (b) inhibition by QV (4.3 μM)

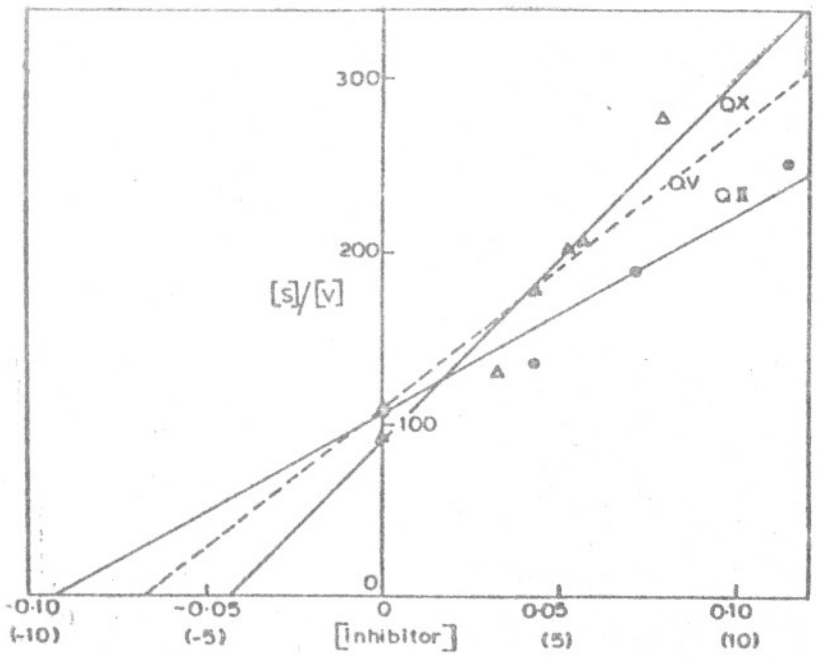


FIGURE 5
Plots of S/V versus inhibitor concentration in μ mol liter^{-1}, for QII, QV and QX. [S]/[V] represents the slope of the Lineweaver-Burke plot. The X-axis scale in parentheses for QII and QV.

presence of the two acetylcholinesterases (Narayanan & Balaram, 1978). This result is probably a consequence of probe binding to a relatively polar site on the enzyme which has minimal effects on fluorescence parameters but allows inhibition of enzyme activity.

QII and QV are less potent inhibitors of HSChE than is QX. This result parallels the data obtained from fluorescence experiments and probably reflects different modes or different sites for the binding of QX on the one hand and QII and QV on the other.

Nuclear magnetic resonance studies

N.m.r. spectroscopy provides a direct method for monitoring protein-small molecule interactions if rapid exchange occurs between the free and complexed states of the ligand (Dwek, 1973). Fig. 6 (spectrum a) shows the region between 2.7 δ and 3.3 δ of the 100 MHz 1H n.m.r. spectrum of QII. The signals at 2.9 δ and 3.1 δ are assigned to the $-N(CH_3)_2$ and $-\overset{+}{N}(CH_3)_3$ protons, respectively, on the basis of chemical shifts and peak intensities. In the absence of protein, both signals are sharp and have linewidths ($\Delta\nu_{1/2}$) of 2.2 Hz. The addition of HSChE to QII (spectrum b, Fig. 6) leads to line broadening, suggesting interaction between probe and protein. This broadening is not accompanied by detectable changes in chemical shift, thus ruling out chemical shift contributions to the enhanced relaxation rates. The observed linewidths are the weighted average of the $\Delta\nu_{1/2}$ values in the bound and free states. Signal broadening results from restrictions in the mobility of the groups at the protein binding site. The broadening of both methyl resonances is indicative of interaction of both

TABLE 2
Inhibition constants (K_i) for the effect of cholinergic probe on enzyme activity

Probe	Enzyme	K_i(M)
QII	HSChE	1.02×10^{-6}
QV	HSChE	6.70×10^{-6}
QX	HSChE	4.40×10^{-6}
QX	Electric eel AChE	1.23×10^{-6}
QX	Bovine erythrocyte AChE	2.50×10^{-6}

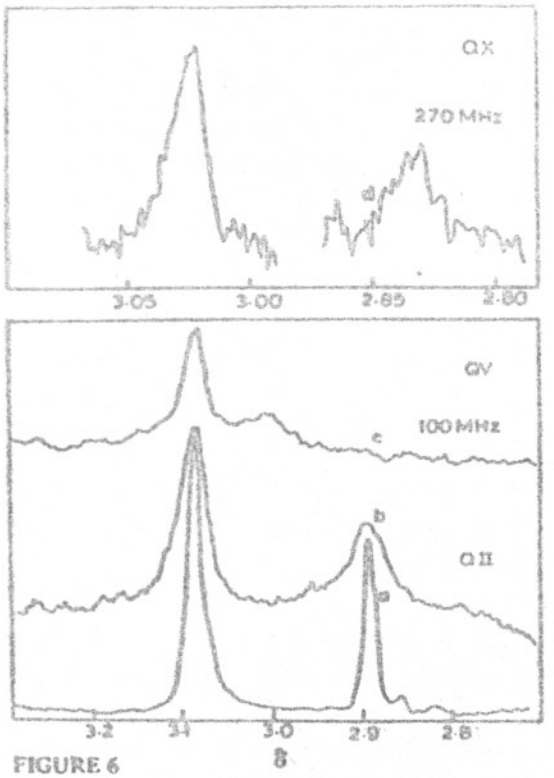
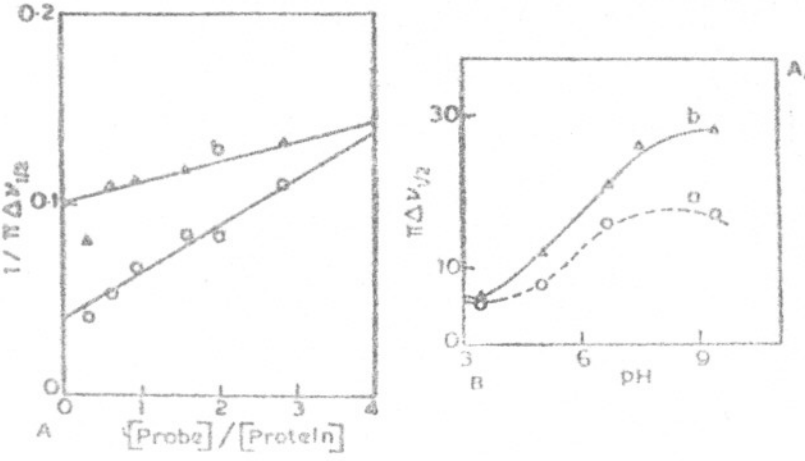


FIGURE 6

The -N(CH$_3$)$_2$ and -$\overset{+}{N}$(CH$_3$)$_3$ signals of the ^{1}H n.m.r. spectra of (a) QII, 0.5 mg/ml, (b) QII, 0.5 mg/ml + HSChE, 3.04 mg/ml, (c) QV, 1 mg/ml + HSChE, 3.0 mg/ml, (d) QX, 1 mg/ml + HSChE, 0.095 mg/ml.

ends of the molecule with enzyme. A significantly larger broadening of the -N(CH$_3$)$_2$ resonance as compared to the -$\overset{+}{N}$(CH$_3$)$_3$ peak is observed over a protein concentration range of 1–3 mg/ml (figure not shown). This is presumably due to greater restrictions to local mobility and consequently closer interactions of the dimethylamino group with HSChE. Fig. 6 (spectrum c) shows that the -N(CH$_3$)$_2$ group of QV broadens to a much greater extent than does the -$\overset{+}{N}$(CH$_3$)$_3$ group in QII, on addition of HSChE. A small downfield shift of about 0.1 δ (10 Hz) is also observed in the presence of protein. Once again the -$\overset{+}{N}$(CH$_3$)$_3$ resonance is less affected. Fig. 6 (spectrum d) shows the methyl resonances of QX in the presence of HSChE. Considerable broadening of both sets of resonances is observed. It may be noted that the spectra were obtained at much lower protein concentrations. This was necessitated by the very marked line broadening in the presence of enzyme. Thus in QX, the longer alkyl chain appears to permit the two ends of the probe molecule to interact with the enzyme surface, much more than in QII and QV.

The variation in the spin-spin relaxation times T_2 ($= 1/\pi\Delta\nu_{1/2}$) of the QV methyl groups as a function of increasing HSChE concentration, is shown in Fig. 7A. A reasonably good linear dependence is obtained and may be extrapolated to provide a crude estimate of the T_2 values for the methyl groups in the probe-protein complex. A much smaller value of T_2 is obtained for the -N(CH$_3$)$_2$ group relative to the -$\overset{+}{N}$(CH$_3$)$_3$ group, confirming the more pronounced immobilization of the former on binding. Fig. 7B shows the effect of varying



A,

FIGURE 7

A, Plot of T_2 (S) versus [probe]/[Protein] for the interaction of QV with HSChE. [Probe] and [Protein] in μ mol liter^{-1} and mg/ml respectively. (a) -N(CH$_3$)$_2$. (b) -$\overset{+}{N}$(CH$_3$)$_3$. B, Plot of $1/T_2$ versus pH for the interaction of QX with HSChE. Probe and protein concentrations are 1 mg/ml and 0.1 mg/ml, respectively. (a) -N(CH$_3$)$_2$, (b) -$\overset{+}{N}$(CH$_3$)$_3$.

pH on the QX-HSChE interaction. Line narrowing of both $-N(CH_3)_2$ and $-\overset{+}{N}(CH_3)_3$ resonances is observed at low pH. A pKa between 6 and 7 is estimated for the titratable group involved in the binding. The reduction in the linewidth is a consequence of the reduced number of bound molecules at low pH. This could arise due to protonation of an anionic residue at the enzyme binding site or due to protonation of the basic dimethylamino function on the probe. The pKa value for the anionic site residue in HSChE is 6.3 (Aldridge & Reiner, 1972), while the protonation of the $-N(CH_3)_2$ group occurs below pH 4 (Himel & Mayer, 1970). Therefore the diminution of linewidth with decreasing pH must be due to the non-availability of a negative charge at the anionic site of HSChE.

The methyl resonances of QX in the presence of HSChE, electric eel AChE and bovine erythrocyte AChE are shown in Fig. 8. At identical probe and protein concentrations, the order of line broadening in the presence of the three proteins is HSChE > eel AChE > erythrocyte AChE. This reflects a stronger interaction of QX with HSChE and is in agreement with the results of fluorescence and inhibition studies. The commercial sample of HSChE used in these studies is known to be a non-homogeneous preparation. However, it has already been shown that the data from fluorescence and inhibition studies reflect the specificity of the probe-HSChE interaction. Further, the probes show negligible interaction with other proteins (Narayanan & Balaram, 1978). Therefore, the results obtained from the n.m.r. studies, which parallel the results obtained from the other techniques, must also arise from specific interactions between the probes and HSChE.

DISCUSSION

The probe structures (Fig. 1A) have a charged quaternary ammonium head group at one end and a hydrophobic naphthalene ring at the other. Binding to the enzyme may therefore involve electrostatic as well as hydrophobic interactions. The site directing action of the $-\overset{+}{N}(CH_3)_3$ group is likely to be similar for QII, QV and QX. The enhancement of binding with increasing alkyl chain length will then presumably reflect the existence of different hydrophobic interactions for the three probes. Quantitation of the probe-protein interaction in terms of binding stoichiometries (n) and dissociation constants (K_D) has been reported earlier (Narayanan & Balaram, 1978). These values have been listed in Table 1 and show that QX interacts with two sites on the protein ($K_D = 6.48 \times 10^{-7}$ M, Site 1 and $K_D = 1.29 \times 10^{-5}$ M, Site 2). The data for QII and QV, however, yield similar values of n and K_D from the two portions of the binding plot. This warrants the assumption that only one type of site characterized by $K_D \sim 10^{-6}$ is being probed by QII and QV. This site may correspond to either of the QX binding sites. The inhibition studies show that QX is a much more potent inhibitor of HSChE than the shorter homologs. It is likely therefore that QX affects enzyme activity by interacting with the main catalytic site

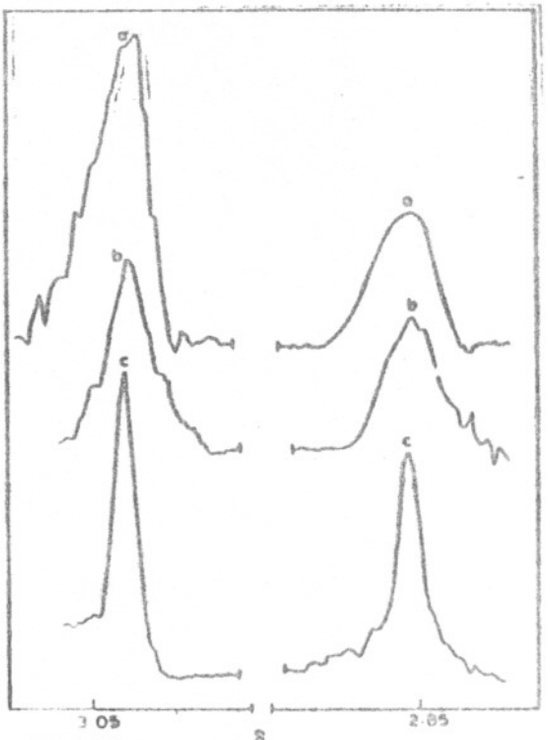


FIGURE 8

The $-N(CH_3)_2$ and $-\overset{+}{N}(CH_3)_3$ signals of the ^1H n.m.r. spectra of QX in the presence of (a) HSChE, (b) electric eel AChE, and (c) bovine erythrocyte AChE. Probe concentration 1 mg/ml. Protein concentration [illegible] mg/ml.

while QV and QII bind to a secondary site and inhibit enzyme activity through an allosteric mechanism. The measurement of binding parameters in the presence of cholinergic inhibitors (Figs. 2,3, Table 1) shows that the binding parameters of QV remain largely unaffected in the presence of HM, DM, CC and TC. However, the addition of CC and DM causes a reduction in the non-linearity of the QX binding plot, and parameters corresponding to Site 2 alone can be obtained. Under identical conditions, two sets of binding parameters can be obtained for QX in the presence of HM and TC.

Carbamylcholine causes inhibition of HSChE activity by binding covalently to the main catalytic site of the enzyme (Augustinsson *et al.*, 1959). Therefore, the abolition of QX binding to Site 1 in the presence of CC arises presumably from the displacement of bound probe from the main catalytic site. The binding of QV to HSChE is not affected by the addition of CC, indicative of probe interaction with a site other than the main catalytic site. The inhibition of HSChE by DM and TC involves the formation of enzyme-inhibitor-substrate (EIS) complexes, implying distinct sites on the enzyme for inhibitor and substrate (Fornasiero 1975*a*, *b*). However, HM, DM and TC are likely to bind with varying affinities to the main catalytic site. The similarity in the effects of the four inhibitors on QV binding and the small changes in probe binding parameters suggest that QV and the inhibitors bind at distinct, separate sites on the enzyme and that simultaneous binding of QV and inhibitor to the enzyme is possible.

The binding parameters listed in Table 1 show that DM and CC abolish the binding of QX to Site 1, but have practically no effect on QX binding to Site 2. Thus while site 1 is presumably the main catalytic site of the enzyme, Site 2 must be a peripheral binding site on the enzyme. An examination of the K_D values characterizing the binding of QX to site 2 in the presence and absence of inhibitors, shows that there are no significant changes in binding affinity. Thus, probe binding to Site 2 seems unaffected by inhibitor occupancy of Site 1. It is significant that the results obtained for the binding of QX to Site 2 parallel those obtained for QV binding to HSChE. This is possibly a consequence of a similarity in the two sites. It is also of interest to note that the blue shift observed for QX bound to HSChE is also seen in the presence of CC (Fig. 1B), despite a drastic decrease in intensity. This further supports the view that the inhibitor acts by direct displacement of QX and not by alteration of the microenvironment of the fluorophore.

The enzyme inhibition data (Table 2) clearly establish that probe binding affects the active site of the enzyme. The K_i values obtained for QII and QV are within an order of magnitude of the value obtained for the related compound 1-(5-dimethylaminonaphthalene-1-sulfonamido) propane-3-trimethylammonium iodide (I) which has a $K_i = 6.3 \times 10^{-6}$ M. Both I and 1-(5-dimethylaminonaphthalene-1-sulfonamido) propane-3-*N*, *N*-dimethylamine (II) are competitive inhibitors of serum ChE. I inhibits AChE activity while II has no effect on this enzyme (Himel *et al.*, 1970). The inhibition of HSChE by *N*-methyl acridinium iodide is characterized by a K_i value of 5.3×10^{-8} M (Chan *et al.*, 1974) nearly equal to the QX value. QX is a much more effective inhibitor of HSChE than the shorter homologs, QII and QV. This result agrees with the fluorescence data and arises, in all probability, from different interactions with the protein.

QX inhibits the activity of AChE from electric eel and bovine erythrocytes, but to a lesser extent than HSChE. The n.m.r. spectra in the presence of the three enzymes (Fig. 8) show greater broadening of the QX methyl signals in the presence of HSChE but are still indicative of interaction between QX and the acetylcholinesterases. However, the fluorescence parameters of QX were not significantly altered in the presence of AChE. Thus, the fluorescence technique is limited by the fact that not all probe-protein interactions can be monitored by it. Its utility is dependent on the environment of the probe binding site.

The results of the n.m.r. experiments suggest a closer interaction of the $-N(CH_3)_2$ group with the protein and consequently greater broadening of this signal relative to the $-\overset{+}{N}(CH_3)_3$ resonance. The slightly less efficient relaxation of the $-\overset{+}{N}(CH_3)_3$ group may arise from the availability of local motional modes for these

methyl groups. The extent of broadening of the signals is proportional to aklyl chain length. In the case of QX, both ends of the molecule interact to a considerable extent with the protein.

A model for the interaction of QV and QX with HSChE, which is compatible with the results obtained from fluorescence, kinetic and n.m.r. experiments, has been presented in Fig. 9. *A* and *E* denote the anionic and esteratic subsites, *P* is a peripheral binding site while *H* represents the hydrophobic area near the anionic subsite. The fluorescence data has shown that QX binds to two sites on the protein over the range of concentrations studied. The high affinity site of QX, denoted earlier as Site 1, is likely to be the *A* site. This is borne out by the diminution in QX fluorescence on the addition of active site inhibitors like carbamylcholine and eserine. Further, the inhibition characteristics of QX suggest competitive inhibition of the enzyme.

The large differences in the results obtained for QV and QX by the three methods suggest that QV binds to a different site on the protein, presumably the *P* site. The binding of QX to the *A* site is probably further strengthened by the interaction between the dansyl moiety and the hydrophobic area, *H*. The length of a fully extended decamethylene chain is about 14 Å (Mooser & Sigman, 1974). Therefore, the hydrophobic area must be at a maximum distance of 14 Å from the *A* site. An interaction of this type is probably not available to QV, accounting for the smaller changes in fluorescence and n.m.r. properties on addition of the protein. The hydrophobic environment of the naphthalene ring of QX leads to the large enhancement of intensity and blue shift in the fluorescence studies and the marked broadening of the $-N(CH_3)_2$ signal in the n.m.r. experiment.

The weaker binding site of QX is likely to be the *P* site, which is also available to QII and QV. This is borne out by the effect of inhibitors on the binding parameters of QV and QX at Site 2. Further, enzyme inhibition and n.m.r. data also suggest a peripheral binding site for QV. Thus, the lengthening of the alkyl chain leads to a change in probe binding characteristics. The binding of QX to the *A* site permits a partial exploration of binding site topography. Structural distinctions between the serum ChE and AChE class of enzymes may also be monitored by the use of such probes.

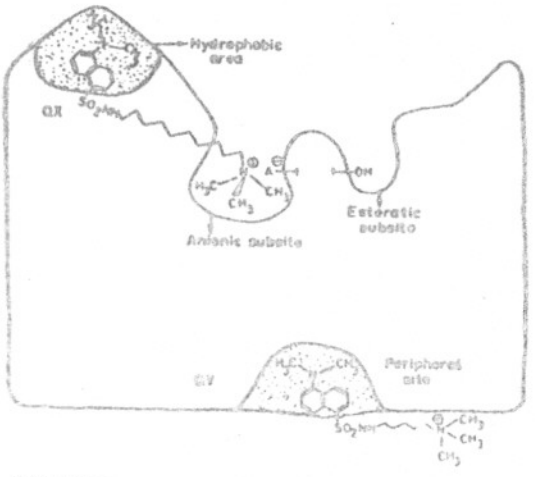


FIGURE 9
Schematic representation of the binding of QV and QX to HSChE.

ACKNOWLEDGMENT

Financial support for this research was received from the Department of Science and Technology, Government of India.

REFERENCES

Aldridge, W.N. & Reiner, E. (1972) In *Enzyme Inhibitors as Substrates - Frontiers of Biology* (Neuberger, A. & Tatum, E.L., eds.), vol. 26, p. 107, North Holland, Amsterdam

Augustinsson, K.-B., Fredriksson, T., Sundwall, A. & Jonsson, G. (1959) *Biochem. Pharmacol.* 3, 68-76

Ayling, J.F. (1973) in *A Guide to Molecular Pharmacology-Toxicology* (Featherstone, R.M., ed.), p. 375, Marcel Dekker, New York

Azzi, A. (1974) *Methods Enzymol.* 32, 234-246

Chan, L.M., Himel, C.M. & Main, A.R. (1974) *Biochemistry* 13, 86-90

Changeux, J.P. (1966) *Mol. Pharmacol.* 2, 369-392

Cohen, J.B. & Changeux, J.P. (1973) *Biochemistry* 12, 4863-4885

Dwek, R.A. (1973) *Nuclear Magnetic Resonance in Biochemistry: Applications to Enzyme Systems*, Clarendon Press, Oxford

Ellman, G.L., Courtney, K.D., Andres, V. & Featherstone

stone, R.M. (1961) *Biochem. Pharmacol.* 7, 88-95
Himel, C.M. & Chan, L.M. (1976) in *Biochemical Fluorescence: Concepts* (Chen, R.F. & Edelhoch, H., eds.), vol. 2, pp. 607-638, Marcel Dekker, New York
Himel, C.M. & Mayer, R.T. (1970) *Anal. Chem.* 42, 130-132
Himel, C.M., Mayer, R.T. & Cook, L.L. (1970) *J. Polymer Sci.* 8, 2219-2230
Himel, C.M., Aboul-Saad, W.G. & Uk, S. (1971) *Agric. Food Chem.* 19, 1175-1180
Kabachnik, M.I., Brestkin, A.P., Godovikov, N.N., Michelson, M.J., Rozengart, E.V. & Rozengart, V.I. (1970) *Pharmacol. Rev.* 22, 355-388
Kamaric, L. (1975*a*) *Biochem. Pharmacol.* 24, 1663-1664
Kamaric, L. (1975*b*) *Croat. Chim. Acta* 47, 287–297
Main, A.R. & Hastings, F.L. (1966) *Science* 154, 400-402
Mooser, G. & Sigman, D.S. (1974) *Biochemistry* 13, 2299-2307
Narayanan, R. & Balaram, P. (1978) *FEBS Lett.* 93, 38-42
Rosenberry, T.L. (1975) *Advan. Enzymol.* 43, 103-218
Scatchard, G. (1949) *Ann. NY Acad. Sci.* 51, 660-672

Address:

Dr. *P. Balaram*
Molecular Biophysics Unit
Indian Institute of Science
Bangalore - 560 012
India