The Interaction of Purified Acetylcholinesterase from Pig Brain with Liposomes

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The binding of pig brain acetylcholinesterase to artificial phospholipid membranes was investigated at different temperatures. Calculation of the thermodynamic parameters revealed a small negative enthalpy change, but a large negative change in the free energy and a large positive change in the entropy on binding. The large entropy change might be interpreted as being responsible for forming the enzyme-membrane complex and was indicative of hydrophobic interactions between lipid and protein. This conclusion would also favour the hypothesis that the enzyme was an integral protein. Further support for this theory was provided by the study of acetylcholinesterase binding to liposomes containing the phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine. Lowering the temperature below the transition temperature or incorporating cholesterol into the liposomes decreased enzyme binding. Both factors could be interpreted as decreasing the fluidity of the hydrocarbon side chains of the phospholipids, causing an increase in bilayer thickness due to closer packing of side chains. This membrane condensation would certainly not favour the binding of integral protein molecules.

Membranes play a vital role in the structure and function of the cell and its organelles, and one of the ways by which membranes can affect cellular metabolism is by interacting with enzymes. For example, some membranes function as the organizing matrix for enzyme systems, and in all cases the micro-environment at or near the membrane surface may modulate the activities of enzymes located in this region. Acetylcholinesterase (EC 3.1.1.7) from a number of sources is always found in association with membranes (Nachmansohn, 1971), and the activity of the enzyme appears to be influenced by this association. For example, lipid-depleted acetylcholinesterase of the erythrocyte membrane shows a marked restoration of activity on adding 3-*sn*-phosphatidylserine, a phospholipid component of membranes (Sihotang, 1976). The fluidity of the membrane also influences the enzyme, and acetylcholinesterase isolated from the erythrocytes of rats fed on corn oil showed allosteric behaviour, whereas the same enzyme isolated from rats fed on lard did not show this behaviour (Bloj *et al.*, 1973; Massa *et al.*, 1975). These authors suggested that the difference observed between the two groups of rats was due to the decrease and enhancement respectively of membrane fluidity. Work in our own laboratory has also demonstrated the effect of the membrane on the acetylcholinesterase from pig cerebral cortex by comparing the soluble and membrane-bound forms

of the enzyme. The acetylcholinesterase of a membrane suspension showed a break in the Arrhenius plot with a transition temperature of 27°C and an activation energy of 8.3 kJ/mol above, and 39 kJ/mol below, this transition temperature. In contrast with this result, the 'naturally soluble' form of the enzyme showed no break in the Arrhenius plot, which might suggest the absence of membrane material. The activation energy in this case was 19 kJ/mol. However, apart from this, the two forms had very similar properties and the differences observed appear to be due to the enzyme being free in solution or associated with the membrane (Plummer *et al.*, 1975).

Most work on acetylcholinesterase has been carried out on a solubilized form of the enzyme separated from membrane structures and free in bulk solution. However, an ultimate understanding of biological events *in vivo* can only be gained the closer the experimental conditions are to the situation that exists *in vivo*, and this present work is concerned with how certain properties of acetylcholinesterase are modified when the enzyme is immobilized. Several methods have been used artificially to immobilize enzymes, such as binding to CNBr-activated Sepharose (Axen *et al.*, 1969), occlusion in polyacrylamide gels (Bernfeld & Wan, 1963) or covalently linking them to polymaleinic anhydride (Alsen *et al.*, 1975). In this present study we have chosen to investigate the interaction of acetylcholinesterase with a phospholipid bilayer in the form of liposomes, as this resembles more closely the situation of the enzyme *in vivo* and has the added

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advantage that the composition of the liposomes can be varied and controlled.

Experimental

Materials

The sources of pig brains and the reagents used for the solubilization and assay of acetylcholinesterase were the same as described previously (McIntosh & Plummer, 1973). Phospholipids used in the preparation of liposomes were obtained from egg yolk (Lipid Products, Nutfield, Surrey, U.K.); stearylamine (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) was recrystallized from ethanol. All other reagents were of analytical grade and solutions were prepared in glass-distilled water.

Methods

Colorimetric assay of acetylcholinesterase. For the binding studies, the enzyme activity was determined at 30°C and pH 8.0 by the colorimetric method of Ellman *et al.* (1961) with acetylthiocholine iodide as substrate.

pH-stat assay of acetylcholinesterase. For the Arrhenius plots, the acetylcholinesterase activity was determined between 5 and 50°C at pH 7.9 by using the pH-stat (McIntosh & Plummer, 1975).

Estimation of protein. Protein was assayed by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. A gelatinous precipitate forms in the presence of Triton X-100, but this interference can be overcome by centrifuging the precipitate (1000g for 5 min) and incorporating Triton X-100 in the reagent blank and standard.

Preparation of the purified enzyme. Acetylcholinesterase was solubilized by extraction of the pig brain with 1% (w/v) Triton X-100 and purified by affinity chromatography. The ligand used in the affinity column was 1-methyl-9-[*N*^β-(ε-aminohexanoyl)-β-aminopropylamino]acridium bromide hydrobromide (Dudai & Silman, 1974) and was linked to Sepharose 4B by the CNBr method of Axen *et al.* (1967). The brain extract was added to the column and eluted with a standard elution buffer of 0.03 M-sodium phosphate, pH 7.0, containing 0.1% (w/v) Triton X-100, until protein could no longer be detected in the fractions. The acetylcholinesterase activity was then eluted with the standard buffer containing the competitive inhibitor decamethonium bromide (10 mM). The column was prepared for further use by washing with 6 M-guanidine hydrochloride followed by a large volume of the standard elution buffer.

The enzyme was assayed by the method of Ellman *et al.* (1961) after dialysis of the fractions for 72 h against three changes of 2 litres of the elution buffer.

No acetylcholinesterase activity was detected in the eluate until the wash with 10 mM-decamethonium bromide, when 44% of the activity was obtained in the peak with a specific activity of 148 μmol/min per mg of protein and a purification of 900-fold. Detergent was present throughout the purification and the acetylcholinesterase preparations used in the following experiments contained 0.1% (w/v) Triton X-100.

Preparation of liposomes. The phospholipids were dissolved in chloroform in a round-bottomed flask and the solvent was removed with a rotary evaporator. When all traces of the chloroform had gone, 4 ml of sodium phosphate buffer (0.05 M, pH 7.4) was added together with several glass balls, the solution was flushed with N₂, then agitated for 10 min, during which time the phospholipids vesiculated to form liposomes. Details of the exact composition of each batch of liposomes are given in the Results section.

Formation of the enzyme-liposome complex and its assay. An 0.2 ml sample of liposomes was mixed with 0.8 ml of sodium phosphate buffer (0.05 M, pH 7.4) and 0.1 ml of 5,5'-dithiobis-(2-nitrobenzoate) prepared as for the Ellman *et al.* (1961) assay of acetylcholinesterase. The purified enzyme (5 μl) was then added, thoroughly mixed and incubated at 25°C for 1 h. The enzyme reaction was started by adding acetylthiocholine iodide in various concentrations and following the change in absorbance in a Perkin-Elmer SP256 dual-wavelength spectrophotometer. To minimize artifacts caused by light-scattering, the beam was selected in the dual-wavelength mode, with the sample wavelength set at 412 nm and the reference wavelength at 452 nm.

Binding studies. The method used was based on that of Redwood & Patel (1974). Liposomes were prepared as described above and 1 ml samples (approx. 2 μmol) incubated with 1–20 μl of purified acetylcholinesterase in 7 ml of sodium phosphate buffer (0.05 M, pH 7.4) for 1 h at 4 and 25°C. The liposomes were then sedimented at 100 000g for 40 min in a centrifuge and resuspended in 1 ml of buffer. The supernatants and suspensions were assayed for acetylcholinesterase activity as described above and the enzyme activity bound per μmol of phospholipid calculated from a knowledge of the amount of phospholipid used in the preparation of the liposomes. Results were presented in the form of Scatchard plots and the association constant for binding calculated (Scatchard, 1949).

Results

Effect of liposomes on the *K_m* of acetylcholinesterase

Positively charged liposomes (85% 3-*sn*-phosphatidylcholine, 15% stearylamine). The Michaelis constant in the absence of liposomes was 44 μM-acetylthiocholine and this increased to 150 μM when the enzyme

was bound to positively charged liposomes (Fig. 1). When Triton X-100 up to a final concentration of 1% (w/v) was added to the liposome complex, the K_m was lowered towards that of the free enzyme, although it never quite reached $44\mu\text{M}$. The V_{max} was not significantly altered by binding to the liposomes.

Negatively charged liposomes (70% 3-*sn*-phosphatidylcholine, 30% myristic acid). Addition of negatively charged liposomes to purified acetylcholinesterase caused the K_m to fall from 44 to $29\mu\text{M}$ (Fig. 1). As with the positively charged liposomes, the V_{max} was unchanged and addition of 1% Triton X-100 to the liposome-enzyme complex brought the K_m back to a value approaching $44\mu\text{M}$.

Temperature and the binding of acetylcholinesterase to liposomes. The association constants (K) at 25°C calculated from the slopes of the Scatchard plots (Fig. 2) were $11.0 \times 10^4 \text{M}^{-1}$ for the positively charged liposomes and $3.4 \times 10^4 \text{M}^{-1}$ for the negatively charged liposomes. These association constants changed very little when measurements were made at 4°C , at which temperature K values of $14 \times 10^4 \text{M}^{-1}$ and $4.0 \times 10^4 \text{M}^{-1}$ were recorded for the positive and negative liposomes respectively (Table 1). The apparent free-energy change for the adsorption was calculated from the equation:

$$\Delta G^0 = -RT \ln K$$

Fig. 1. Double-reciprocal plots showing the effect of liposomes on the activity of acetylcholinesterase. ○, Control (no liposomes); ■, positively charged liposomes; ●, negatively charged liposomes.

where R is the gas constant, T the temperature (K) and K the association constant. Very similar values were obtained in the range -24 to -29kJ/mol for both groups of liposomes at 25°C and also 4°C (Table 1).

The standard change in enthalpy (ΔH^0) was calculated from the integrated Van't Hoff equation:

$$\ln \frac{K_2}{K_1} = \frac{\Delta H^0}{R} \cdot \frac{T_2 - T_1}{T_2 T_1}$$

where K_1 and K_2 are the association constants at temperatures T_1 and T_2 . This was found to be small and negative, with a value of -8kJ/mol for the positively charged liposomes and -5kJ/mol for the negatively charged liposomes.

Acetylcholinesterase and liposomes of 50% 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 50% myristic acid

Temperature and enzyme activity. The phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine shows a phase change at 24°C (Redwood & Patel, 1974).

Fig. 2. Scatchard plots for the binding of acetylcholinesterase to positively (○) charged and negatively charged (●) liposomes at 25°C .

Table 1. Thermodynamic parameters for the binding of acetylcholinesterase to liposomes

The association constants for the binding of acetylcholinesterase to positively and negatively charged liposomes at 4 and 25°C were obtained from the Scatchard plots (Fig. 2). The thermodynamic parameters were then calculated from these constants by using the formulae referred to in the text.

Liposomes		Temperature ($^\circ\text{C}$)	Association constant (M^{-1})	ΔH^0 (kJ/mol)	ΔG^0 (kJ/mol)	ΔS^0 ($\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$)
Composition	Charge					
Stearylamine (15%) and phosphatidylcholine (85%)	+	25	11×10^4	-8	-29	+70
		4	14×10^4	-8	-27	+69
Myristic acid (30%) and phosphatidylcholine (70%)	-	25	3.4×10^4	-5	-26	+70
		4	4.0×10^4	-5	-24	+69

When acetylcholinesterase was bound to liposomes containing equal parts of this phospholipid and myristic acid, the Arrhenius plot of the enzyme activity showed a phase change at 21°C with activation energies of 7.3 kJ/mol above this temperature and 14.4 kJ/mol below 21°C. Incorporation of cholesterol (1%, mol/mol) into the liposomes abolished the phase change and increased the activation energy to 20 kJ/mol (Fig. 3).

Temperature and enzyme binding. The effect of binding acetylcholinesterase to these liposomes on either side of the transition temperature for 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine was examined. At 25°C K was $0.95 \times 10^4 \text{ M}^{-1}$. Table 2 shows the activity of acetylcholinesterase bound to the liposomes at 25 and 4°C, and it is clear from these results that lowering the temperature below the transition temperature or incorporating cholesterol in the liposomes decreases the binding of the enzyme below that found for liposomes without cholesterol at 25°C.

Fig. 3. Arrhenius plots of acetylcholinesterase adsorbed to liposomes without (○) and with (●) cholesterol

Discussion

The effects caused by the charged liposomes on the K_m of acetylcholinesterase can be explained by interpreting the pH and substrate-concentration changes at a surface in relation to the bulk phase. Alterations in K_m may be due to changes in the substrate affinity, although this is not necessarily so. However, if the assumption is made that the enzyme-substrate affinity is affected in the way discussed then it is possible to explain the changes in K_m when the enzyme is bound to the liposomes.

Acetylcholinesterase has an anionic or negative centre in its active site (Wilson & Bergmann, 1950a) and this area of the molecule is responsible for orientating the ester bond in the acetylcholine over the esteratic site by binding the quaternary nitrogen atom of the choline moiety. On the basis of the charge carried by the substrate and its effect on enzyme binding, ionic forces appear to play an important role in the association of acetylcholinesterase and its substrate (Hestrin, 1949; Wilson & Bergmann, 1950b). These workers found a decreased binding of acetylcholine and other positively charged substrates to the enzyme as the pH was raised. Now at a positively charged lipid bilayer there would be a repulsion of H^+ from the surface, so that the pH at the surface would be higher than that in the bulk phase (Davies & Rideal, 1963). On this basis therefore, positively charged liposomes would be expected to lower the affinity of the bound enzyme for its natural substrate. With parallel reasoning, the lower pH at the surface of the negatively charged liposomes would be expected to increase the enzyme-substrate affinity. Another factor affecting this affinity is the unequal distribution of substrate molecules between the surface phase and the bulk phase. A positively charged surface would repel the positively charged acetylcholine molecules so that the concentration of substrate molecules close to the bound enzyme would be decreased. This would have the effect of lowering the affinity of the enzyme for its substrate compared with that found for the enzyme

Table 2. Effects of cholesterol and temperature on the binding of acetylcholinesterase to liposomes

Liposomes were prepared and the binding of acetylcholinesterase to these structures measured as described in the text. The temperatures at which the measurements were carried out, namely 25 and 4°C, were selected as they are above and below the transition temperature for acetylcholinesterase bound to liposomes of 50% myristic acid and 50% 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (Fig. 3). The effect of cholesterol on the degree of binding was also investigated.

Liposome composition (%)			Temperature (°C)	Enzyme activity bound (nmol/min per mol of phospholipid)
Myristic acid	1,2-Dimyristoyl- <i>sn</i> -glycero- 3-phosphocholine	Cholesterol		
50	50	0	25	5.3
50	50	0	4	2.9
49.5	49.5	1	25	1.9
49.5	49.5	1	4	2.0

in free solution. Similarly for a negatively charged surface, the enzyme-substrate affinity would be increased (Goldstein *et al.*, 1964). The positively charged liposomes did indeed substantially raise the K_m for acetylcholine to $150\ \mu\text{M}$, from $44\ \mu\text{M}$ found for the enzyme in free solution. Conversely when the enzyme was bound to the negatively charged liposomes the K_m was decreased to $29\ \mu\text{M}$ (Fig. 1). In both of these cases the addition of Triton X-100 abolished the change in K_m , thus underlining the effect of the polyelectrolyte nature of the liposomes on the hydrolysis of acetylcholine. The $V_{\max}$ for acetylcholinesterase was not affected by the liposomes, which suggests that no inactivation of the enzyme took place and that the integrity of the enzyme was maintained when bound to the surface.

At neutral pH values most proteins are negatively charged, so that the enzyme would be expected to bind more readily to the positive rather than the negative liposomes, and the results obtained for the association constants (K) appear to bear this out (Table 1). However, an analysis of the thermodynamic parameters calculated from the experimental results suggest that the forces involved in the binding of acetylcholinesterase to the liposomes are predominantly hydrophobic in nature rather than electrostatic. The association constants were of the same order of magnitude for both positive and negative liposomes and changed only slightly when the temperature was lowered from 25 to 4°C. The apparent enthalpy change (ΔH°) calculated from the Van't Hoff equation was therefore small and amounted to only -8 kJ/mol for the positive liposomes and -5 kJ/mol for the negative liposomes (Table 1). The high affinity of the enzyme for both types of liposomes is reflected in the favourable free-energy change of adsorption (ΔG°) and since the enthalpy change is small, there must be a large change in the entropy (ΔS°) on binding. The Gibbs-Helmholtz equation was used to calculate the entropy change:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

and this amounted to $+70\text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$ at 25°C and $+69\text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$ at 4°C for both positive and negative liposomes. This large positive entropy change after the adsorption of acetylcholinesterase to liposomes suggests that the association is predominantly hydrophobic (Kauzmann, 1959; Schachman, 1963). Redwood & Patel (1974) also obtained a large entropy change when studying the binding of adenosine triphosphatase to liposomes and interpreted this as indicating that the interaction between the lipid and protein molecules was hydrophobic in nature. For brain acetylcholinesterase, the presence of predominantly hydrophobic binding would support the idea that the enzyme is an integral protein interacting with the apolar interior of

membranes *in vivo*, an idea supported by the necessity to use detergents to bring the enzyme into solution (Jackson & Aprison, 1966; Ho & Ellman, 1969; McIntosh & Plummer, 1973). Further support for the suggestion that brain acetylcholinesterase is an integral rather than a peripheral protein (Singer & Nicolson, 1972) comes from studies on the effect of temperature on the activity of the enzyme. Arrhenius plots of the membrane-bound enzyme from pig brain clearly showed a break at 27°C, with a large increase in the energy of activation from 8.3 to 39 kJ/mol as the temperature fell below the transition temperature (Plummer *et al.*, 1975). The break could be due to a change in the physical state of the membrane lipids from a crystal to a liquid-crystal form (Raison, 1973). This seems likely, as the transition temperature was abolished on addition of detergent and was not seen in the naturally soluble form of the enzyme (Plummer *et al.*, 1975). However, other interpretations are possible for breaks in Arrhenius plots (Dixon & Webb, 1964) and it was thought that if the solubilized enzyme was bound to an artificial membrane and still showed a phase change characteristic of the constituent phospholipids then it would provide further evidence for the enzyme being an integral protein.

The phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine has been reported to undergo a phase transition at about 24°C (Redwood & Patel, 1974), and the transition temperature found for the activity of acetylcholinesterase bound to liposomes composed of this phospholipid and myristic acid was in the same range, at approx. 21°C (Fig. 3). The incorporation of cholesterol into a lipid bilayer decreases the motional freedom of the methylene groups of the hydrocarbon chains (Chapman & Penkett, 1966) and increases the bilayer thickness by closer packing of the chains (Phillips, 1972). The condensing effect of cholesterol thus favours the crystal form of the lipid bilayer. The incorporation of cholesterol into liposomes prepared from 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine appears to condense the lipid bilayer so as to keep it in its crystal form even above the transition temperature characteristic of the phospholipid. This is shown by the straight-line Arrhenius plot obtained when acetylcholinesterase is incorporated into these liposomes and the high energy of activation of 20 kJ/mol compared with 7.3 kJ/mol for the 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine liposomes above the transition temperature (Fig. 3). The incorporation of cholesterol into liposomes of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine also decreased the binding of acetylcholinesterase (Table 2). Papahadjopoulos *et al.* (1973) also reported a decrease in protein binding to phospholipid liposomes after the incorporation of cholesterol.

The break in the Arrhenius plots obtained for the

membrane-bound enzyme (Plummer *et al.*, 1975) therefore probably represents a true phase change in the lipids of the integral membrane and further supports the suggestion that brain acetylcholinesterase is intimately associated with the membrane *in vivo*.

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