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RECONSTITUTION OF HUMAN ERYTHROCYTE MEMBRANE ACETYLCHOLINESTERASE IN PHOSPHOLIPID VESICLES

ANALYSIS OF THE MOLECULAR FORMS BY CROSS-LINKING STUDIES

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Summary

Unilamellar lipid vesicles were formed upon removal of Triton X-100 with Amberlite XAD-2 from a mixture of egg phosphatidylcholine and Triton-solubilized pure human erythrocyte membrane acetylcholinesterase. A majority of large (230 nm diameter) vesicles together with a minor population of smaller (30 nm diameter) structures were observed in freeze-fracture electron micrographs. Reconstitution experiments performed with [*phenyl*-^{3}H(n)]-Triton X-100 showed that only one detergent molecule per 600 lipid molecules was present in the vesicles. Density gradient centrifugation showed co-sedimentation of acetylcholinesterase with the lipid vesicles. About 60% of the incorporated enzyme was directed towards the vesicle exterior and could be partially degraded by papain. Mainly dimeric acetylcholinesterase was found when the reconstituted or, alternatively, the lipid-free but Triton-solubilized enzyme were cross-linked with glutaraldehyde. Aggregates were observed when the detergent-depleted oligomeric forms of the enzyme were cross-linked. The results thus indicate that mainly the dimeric enzyme form is present in a phospholipid environment.

Introduction

Human erythrocyte membrane acetylcholinesterase can be purified by affinity chromatography of Triton X-100-solubilized red blood cells [1—3].

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In density gradient centrifugation, an apparently homogeneous enzyme form with an *s* value of 6.5 is obtained. Due to hydrophobic interactions, this enzyme aggregates in the absence of detergent to multiple molecular forms with molecular weights from 500 000 to 1 137 000 [4]. Although the isolated enzyme and some of the oligomeric forms have been characterized [1—4] nothing is known about the state of aggregation of acetylcholinesterase in a lipid environment.

Similar problems have been encountered with other membrane-bound proteins, such as the band 3 polypeptide of the erythrocyte membrane [5]. To elucidate the subunit composition of this protein, the cross-linking technique has been applied [6]. This method provides useful information about the composition and symmetry of oligomeric proteins [6—8].

In the present study, incorporation of purified human erythrocyte membrane acetylcholinesterase into phospholipid vesicles was achieved by a detergent-depletion method. The state of aggregation of the reconstituted enzyme was investigated by cross-linking experiments.

Materials and Methods

Outdated erythrocyte sediments were obtained from the Central Laboratory of the Swiss Red Cross Blood Transfusion Service.

Triton X-100 was purchased from Bender and Hobein AG, Zürich, Switzerland. [*phenyl*-^{3}H(n)]Triton X-100 (1 mCi/ml) was from New England Nuclear, Boston, U.S.A. Amberlite XAD-2, acrylamide, *N,N'*-methylene bisacrylamide and Coomassie brillant blue G-250 were obtained from Serva, Heidelberg, F.R.G. Beef liver catalase was a product from Boehringer, Mannheim, F.R.G. and bovine serum albumin was purchased from Poviet Producten, Amsterdam, The Netherlands. Papain was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. L-Phosphatidylcholine from egg yolk was from Koch Light Laboratories, Colnbrook, Bucks, U.K. Protein and vesicle samples were concentrated in collodion bags SM 13200 from Sartorius Membranfilter GmbH, Göttingen, F.R.G. Glutaraldehyde (25% solution in H_2O under N_2, purissimum) for electron microscopy was obtained from Fluka AG, Buchs, Switzerland. Dimethylsuberimidate was a gift from Dr. J. Hajdu, Budapest, Hungary. Edrophonium chloride was a gift from Hoffmann-La Roche Company, Basel, Switzerland. All other reagents were analytical grade products either from Fluka AG, Buchs, Switzerland or Merck, Darmstadt, F.R.G.

Acetylcholinesterase preparation. The enzyme was prepared from outdated human erythrocyte sediments. The purification was performed essentially as described in Ref. 1 with the following modifications. The elution buffer for affinity chromatography contained edrophonium chloride instead of decamethonium bromide and a Triton X-100 concentration of 0.05% instead of 1.0%.

Enzyme assays and protein determination. Acetylcholinesterase activity was determined according to the method of Ellman et al. [9]. The 3 ml assay mixture contained 1 mM acetylthiocholine, 0.125 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 0.05% Triton X-100 in 100 mM phosphate buffer, pH 7.4. Catalase was determined following the procedure of Aebi [10]. Protein concen-

tration was measured according to the method of Lowry et al. [11], using bovine serum albumin as standard protein.

Preparation of Amberlite XAD-2. The polystyrene resin, Amberlite XAD-2, was pretreated as described in Ref. 12 and stored in water containing 0.02% NaN_3 (w/v) at 4°C.

Incorporation of purified acetylcholinesterase into phospholipid vesicles. Egg phosphatidylcholine (40 mg) was dried with a stream of N_2 and then kept under vacuum for 1 h. The dried lipids were dissolved in 10 ml (200 mM) triethanolamine-HCl buffer, pH 8.5, containing 0.5% Triton X-100 (w/v). Pretreated Amberlite XAD-2 (3 g wet wt.) was added and the mixture was gently stirred for 1 min. Acetylcholinesterase in triethanolamine-HCl buffer, pH 8.5, containing 1% Triton X-100 was then added to a final concentration of 20 or 200 μg protein per ml. The solution was stirred for 24 h at 4°C. Amberlite XAD-2 was then removed by filtering the solution through glass wool. The filtrate was stored at 4°C under N_2.

Freeze-fracture electron microscopy. The samples were frozen essentially as described by Moor et al. [13]. Fracturing was carried out in a Balzers BAF 300 freeze-fracture apparatus. The replicas were examined in a Philips EM 301 electron microscope, operated at 100 kV.

Cross-linking experiments. The monomeric form of glutaraldehyde in aqueous solution was stabilized by addition of glacial acetic acid (1 μl/ml) [14]. The acidic solution was stored under N_2 until use. The cross-linking reaction was performed with 20 mM glutaraldehyde in 200 mM triethanolamine-HCl buffer, pH 8.5, for 1 h at 4°C. Cross-linking was stopped by adjusting the pH of the solution to 7.0 or, alternatively, by addition of hydroxylamine to a final concentration of 200 mM. The cross-linked protein samples were prepared for electrophoresis by immediate dialysis against 10 mM Tris-acetate buffer, pH 7.0.

Cross-linking experiments with 20 mM dimethylsuberimidate were performed under conditions identical to those described above. The cross-linking reaction was started by addition of the protein-containing solutions to the solid imidate.

Sepharose 4B chromatoraphy. A Sepharose 4B column (2.0 × 35 cm) was equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl and 0.02% NaN_3. To minimize the loss of reconstituted material, a suspension of sonicated egg phosphatidylcholine (10 mg) was passed through the column prior to the application of enzyme-containing vesicles. Samples of reconstituted vesicles (2 ml each) were then applied and fractions of 1.9 ml were collected. Acetylcholinesterase activity in the presence of 0.05% Triton X-100 and lipid phosphorous [15] were determined in each fraction.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. The electrophoresis and staining methods used are described in Ref. 16. Prior to electrophoresis, protein samples were dialysed overnight against 10 mM Tris-acetate buffer, pH 7.0, at 4°C and, if necessary, concentrated in collodion bags to protein concentrations of approx. 10 μg per 100 μl. Each sample was mixed with sucrose (final concentration about 10%) and 15 μl of a solution containing 10% sodium dodecyl sulfate (SDS), 10% mercaptoethanol and 0.01% bromophenol blue in water. The samples were heated for 10 min in a boiling-water

bath and immediately layered onto the gels. Electrophoresis was carried out at 0.4 mA/mm^2 and 12°C for approx. 3 h.

Papain digestion experiments. 500 μg of papain were activated for 20 min at 25°C in 100 μl of a buffer containing 50 mM Tris, 5 mM cysteine-HCl and 2 mM EDTA, pH 7.5, which had been saturated with N_2 [17]. Vesicle samples containing 9—18 μg of protein in 10 mM Tris-HCl, 0.1 M NaCl, pH 7.4, were incubated with 40 μg of activated papain at room temperature. Analogous experiments were performed with isolated enzyme in 10 mM Tris-HCl, 0.1 M NaCl, 0.1% Triton X-100 (w/v), pH 7.4.

Results

Characterization of vesicles

Removal of Triton X-100 from detergent/lipid/protein mixed micelles with Amberlite XAD-2 resulted in the formation of vesicles which were visualized by freeze-fracture electron microscopy (Fig. 1). A majority of large, lipid bilayer vesicles with a diameter of approx. 230 nm was detected, together with a minor fraction of small (30 nm) vesicles. Column chromatography of the vesicle preparation showed a major peak of lipid phosphorous in the void volume of the column (Fig. 2) together with most of the acetylcholinesterase activity and some residual Triton X-100. A further but considerably smaller

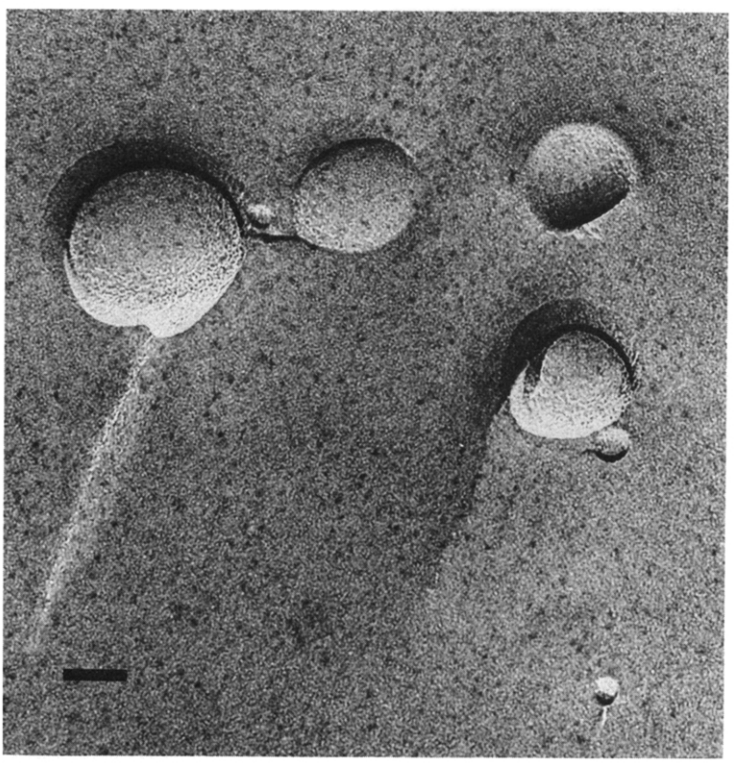

Fig. 1. Freeze-fracture electron microscopy of reconstituted vesicles. Electron microscopy was carried out as described in Materials and Methods. The bar represents 100 nm.

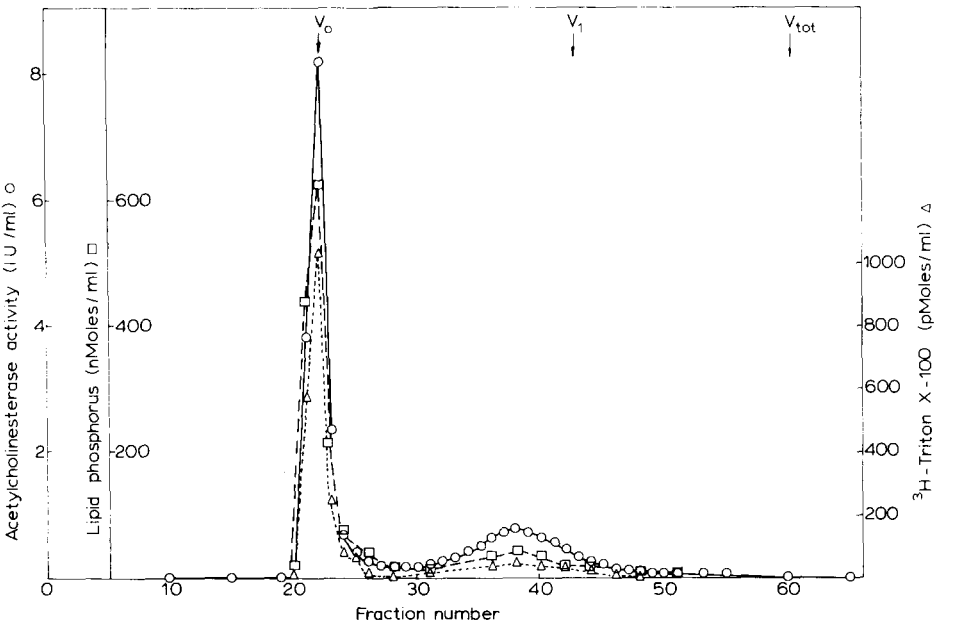

Fig. 2. Gel filtration of reconstituted acetylcholinesterase on Sepharose 4B. Vesicles were prepared as described in Materials and Methods from a solution containing [*phenyl*-^{3}H(n)]Triton X-100. V_0 and V_{tot} of the column were determined with dextran blue and ferricyanide, respectively. V_1 indicates the elution volume of aggregated, non-reconstituted acetylcholinesterase.

amount of enzyme, lipid phosphorus and Triton X-100 was eluted within the separation range of the column as a second peak. Obviously, the material in this smaller peak corresponded to the small vesicle fraction observed in electron micrographs. The fraction with large vesicles, obtained in column chromatography, contained 625 nmol lipid/ml and a residual amount of 1.03 nmol [*phenyl*-^{3}H(n)]Triton X-100/ml. Thus, the lipid-to-detergent ratio in the reconstituted vesicles was 600 : 1. For the small vesicle fraction, a ratio of 900 : 1 was calculated.

Density gradient centrifugation of the vesicle preparation showed that acetylcholinesterase floated on top of the gradient, together with the lipid phosphorus (Fig. 3). A control experiment confirmed that acetylcholinesterase which was not lipid-associated migrated towards the bottom of the gradient under the conditions employed.

Clear differences between vesicle-associated and free acetylcholinesterase were again apparent in the susceptibility of the enzyme to papain digestion. The results of these experiments are summarized in Table I.

Cross-linking experiments

Enzyme cross-linked with glutaraldehyde in the presence of 0.1% Triton X-100 (dimeric acetylcholinesterase; Ref. 18) showed only one peak of activity (6.5 S) in sucrose density gradient analysis (Fig. 4A). In contrast, detergent-depleted, aggregated acetylcholinesterase treated with glutaraldehyde showed a considerably different pattern (Fig. 4B), with mostly aggregated enzyme forms, although the density gradients were also made in 0.1% Triton X-100. SDS-

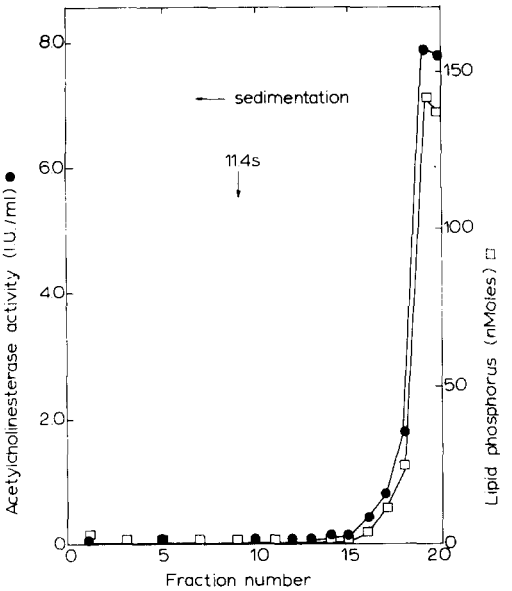

Fig. 3. Sucrose density gradient centrifugation of reconstituted acetylcholinesterase. Reconstituted acetylcholinesterase was analysed on linear sucrose density gradients (5—30% sucrose) made in 10 mM Tris-HCl, 0.1 M NaCl buffer, pH 7.4, with 0.2 mg catalase as reference material. After centrifugation at 200 000 X *g* and 4°C for 15 h, 20 fractions were collected from the bottom.

polyacrylamide gel-electrophoresis confirmed these results. Fig. 5 shows the pattern obtained from the enzyme, cross-linked in the presence of Triton X-100 (gel c). The two main bands observed could be assigned to the monomeric (gel a) and dimeric enzyme forms (gel b). Some minor amounts of oligomers were detected as well. Enzyme cross-linked in the absence of detergent yielded the pattern shown in Fig. 5d, with most of the protein present as high molecular weight forms.

The effect of cross-linking acetylcholinesterase incorporated into phospholipid vesicles (lipid-to-protein ratio (w/w) 100 : 1) with glutaraldehyde was again investigated by density gradient centrifugation (Fig. 4C). Most of the enzyme (88%) is present as the 6.5 S form and only 12% of aggregated acetylcholin-

TABLE I

EFFECT OF PAPAIN-TREATMENT ON ACETYLCHOLINESTERASE ACTIVITY

Incubation with papain was carried out as described in Materials and Methods. Incubation time was 60 min. n.d., not determinable. As shown previously [32], the non-reconstituted 6.5 S enzyme, when assayed in the absence of Triton X-100, becomes rapidly inactivated.

Enzyme treatment	Enzyme activity (%) assayed	
	0.05% Triton X-100 present	Triton X-100 absent
Enzyme untreated	100	n.d.
Reconstituted enzyme untreated	100	57
Enzyme papain-treated	8	n.d.
Reconstituted enzyme papain-treated	64	27

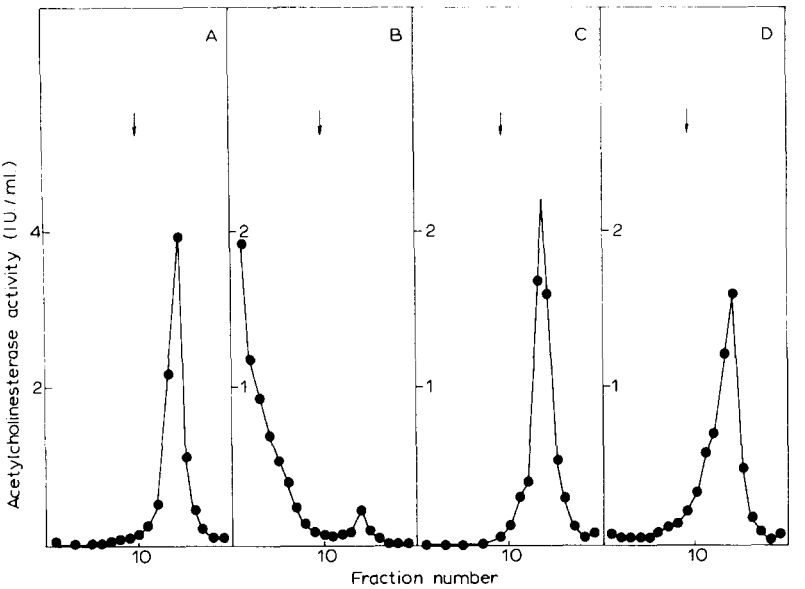

Fig. 4. Sucrose density gradient centrifugation of cross-linked acetylcholinesterase preparations. Analysis was performed on linear sucrose density gradients containing 5—30% sucrose in 10 mM Tris-HCl, pH 7.4, 0.1 M NaCl with 0.1% Triton X-100. The gradients contained enzyme or vesicle samples treated with 20 mM glutaraldehyde: (A) acetylcholinesterase cross-linked in the presence of 0.1% Triton X-100; (B) aggregated acetylcholinesterase cross-linked in the absence of Triton X-100; (C) reconstituted acetylcholinesterase (lipid-to-protein ratio (w/w) of 100 : 1); (D) reconstituted acetylcholinesterase (lipid-to-protein ratio (w/w) of 20 : 1). The position of the marker enzyme catalase (11.4 S) is indicated by an arrow.

esterase could be found. If the lipid-to-protein ratio was changed to 20 : 1 the share of oligomeric enzyme was increased to 34%. The major part, however, was still present as the dimer (66%; Fig. 4D). This finding was again confirmed by gel electrophoresis (Fig. 5, gels e and g, respectively).

With acetylcholinesterase incorporated into vesicles but not treated with glutaraldehyde, the pattern shown in Fig. 5f resulted, analogously to that obtained for the uncross-linked lipid-free enzyme (Fig. 5a). In both cases only monomeric acetylcholinesterase was observed.

TABLE II

DISTRIBUTION OF VARIOUS FORMS OF CROSS-LINKED ACETYLCHOLINESTERASE

Values were calculated from density gradient experiments shown in Fig. 4. Values are given in % of total activity applied onto individual gradients.

Density gradient	Enzyme form (%)	
	Dimer	Aggregates
A	100	0
B	9	91
C	88	12
D	66	34

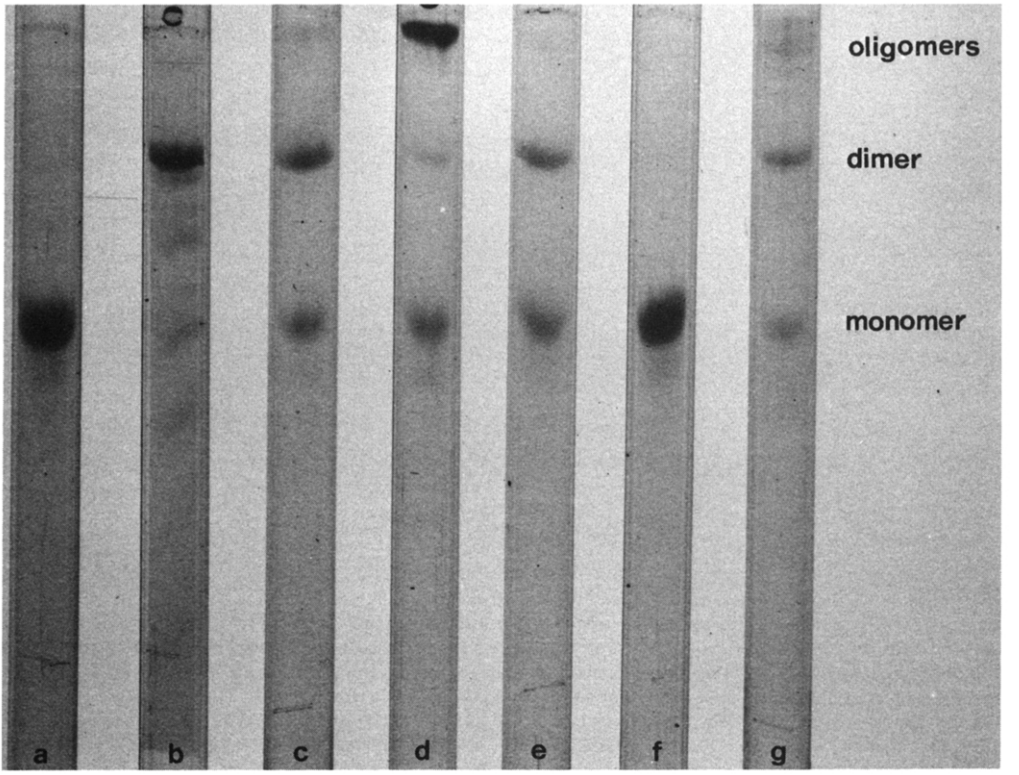

Fig. 5. Sodium dodecyl sulfate gel electrophoresis of acetylcholinesterase. Electrophoresis was carried out as described in Materials and Methods. With one exception (gel b) the protein samples were run under reducing conditions. The gels show: (a) monomeric acetylcholinesterase, (b) dimeric acetylcholinesterase, (c) acetylcholinesterase cross-linked in the presence of 0.1% Triton X-100 with 20 mM glutaraldehyde, (d) aggregated acetylcholinesterase cross-linked with 20 mM glutaraldehyde, (e) reconstituted acetylcholinesterase (lipid-to-protein ratio (w/w) of 100 : 1) cross-linked with 20 mM glutaraldehyde, (f) the same preparation as in e but not cross-linked and (g) reconstituted acetylcholinesterase (lipid-to-protein ratio (w/w) of 20 : 1) cross-linked with 20 mM glutaraldehyde.

A summary of the quantitative distribution of the different acetylcholinesterase forms obtained in density gradient centrifugation is given in Table II.

Discussion

The behavior of vesicle preparations on Sepharose 4B column chromatography showed a complete coincidence of the elution volumes of acetylcholinesterase activity and phospholipid (Fig. 2). The same coincidence was found in sucrose density gradient centrifugation experiments (Fig. 3) whereas, with both methods, the lipid-free enzyme showed a clearly different behavior. The observed change in hydrodynamic properties of the reconstituted enzyme brought about by lipids strongly supports the idea that the enzyme is closely associated with the lipid structures. Similar observations have been considered as evidence for the incorporation of proteins into phospholipid vesilces [19—21].

Electron microscopy results (Fig. 1) give clear evidence for vesicles which,

however, do not show intramembraneous particles. It should be noted though, that this does not exclude the presence of enzyme incorporated into the lipid bilayer, as similar negative results were obtained with glycophorin [22,23]. Further evidence for enzyme reconstitution has been obtained from the finding that the activity of acetylcholinesterase incorporated into vesicles is strongly modulated by the gel-to-liquid-crystalline phase transition of the phospholipid [24]. Consistent with earlier results [21], only part of the incorporated enzyme (57%) was accessible to substrate without disrupting the vesicle structure with detergent (Table I). This part must be oriented towards the outside of the membrane. Upon papain digestion, not more than 53% of this externally oriented acetylcholinesterase was degraded, whereas lipid-free enzyme lost 92% of its activity under the same incubation conditions. Apparently, the lipid-associated enzyme is protected against the attack of proteolytic enzymes. A similar protection has also been shown to occur in myelin basic protein [25] and has been explained by the hydrophobic interactions of the protein with the lipid core. These considerations further strengthen the evidence that acetylcholinesterase closely interacts with the hydrophobic portion of the phospholipid bilayer.

The removal of Triton X-100 by the use of Amberlite XAD-2 resulted in a detergent-to-lipid ratio of 1 : 600 (mol/mol) in the vesicle preparation. This is considerably lower than the value reported by Gerritsen et al. [26] who found a ratio of 1 : 100 when band 3 and other red cell membrane proteins were reconstituted using Bio-Beads SM-2 as detergent-removing resin. It can be calculated that in our case, 7—8 molecules of Triton X-100 are present per molecule of acetylcholinesterase (molecular weight 160 000) after reconstitution at a lipid-to-protein ratio of 20 : 1 (w/w).

To establish the state of aggregation of acetylcholinesterase in phospholipid vesicles, cross-linking experiments were carried out with reconstituted and with lipid-free acetylcholinesterase. Glutaraldehyde was chosen because it is known to be a highly reactive, bifunctional reagent [14,27,28] which is able to form covalent stable bonds with ϵ-lysin groups of proteins [29,30].

As demonstrated earlier, the 6.5 S enzyme is a dimer in the presence of micellar Triton X-100 concentrations [18]. After removal of the detergent the enzyme aggregates to higher molecular weight forms [4]. If these forms are cross-linked and analyzed under dissociating conditions (i.e., in the presence of detergent micelles), forms with *s* values higher than 11 S are found predominantly. The detergent, thus, is no longer able to dissociate the oligomeric species because they now are stabilized by covalent bonds. On the other hand, only the 6.5 S form is found if acetylcholinesterase is cross-linked in the presence of Triton X-100. This behavior indicates that the form of the resulting cross-linked enzyme is the same as the enzyme form before cross-linking. Thus, only intramolecular cross-links are expected [30]. This is further supported by the results of the polyacrylamide gel electrophoresis patterns shown in Fig. 5c and d. The considerable amount of monomeric acetylcholinesterase detectable has to be due to a limited availability of ϵ-lysine groups [31,18]. Under our conditions, neither prolonged incubation with glutaraldehyde nor increased reagent concentrations changed the amount of cross-linked products.

Acetylcholinesterase cross-linked after incorporation into phospholipid

vesicles behaved quite differently (Fig. 4c and d; Fig. 5e and g). The most striking feature is that oligomers larger than dimers are present only to a rather limited extent and that predominantly the 6.5 S form is present (Figs. 4 and 5; Table II). A possible explanation might be that formation of aggregates is prevented by residual Triton X-100 in the vesicles. On the other hand, complete aggregation of the enzyme has been shown to occur at a concentration of 2.5 μg protein/ml [32]. Under these conditions, 16 molecules of Triton X-100 were present per dimer. Thus, it seems highly unlikely that in the reconstituted system enzyme aggregation could have been prevented by only 7 molecules of detergent per dimer at a protein concentration of 200 μg/ml. Apparently, the lipids in the vesicle bilayer interact with the hydrophobic region of the acetylcholinesterase dimer in such a way that aggregation is effectively diminished. This situation is consistent with earlier observations [4] where chaotropic ions, known to influence hydrophobic interactions, have been shown to reduce aggregation of acetylcholinesterase dimers upon removal of detergent. In vesicles prepared at a lipid-to-protein ratio of 20 : 1, up to 34% of the enzyme was present as oligomeric, preferentially tetrameric species. Similar results were obtained with vesicles cross-linked after 1000-fold dilution. Therefore, intervesicular cross-linking can be excluded and it might be assumed that some tetrameric acetylcholinesterase is present in the reconstituted vesicle. On the other hand, one has to consider the fast lateral diffusion of proteins known to occur in artificial and natural membranes [33]. It is possible that, during the incubation period necessary for cross-linking, spatially separated dimers approach each other to form collisional complexes which are then cross-linked by glutaraldehyde. This process may even be augmented by the long chain polymers of glutaraldehyde which are though to be the reactive components [14]. This point is further supported by the finding that cross-linking of reconstituted enzyme with dimethylsuberimidate (chain length of 11 Å, Ref. 34) resulted in dimeric acetylcholinesterase only. This finding was obtained although dimethylsuberimidate cross-linked enzyme aggregates to a higher yield than dimeric acetylcholinesterase in lipid-free systems [18].

Based on a report by Huang and Mason [35] which permits the calculation of the number of phospholipid molecules in a single vesicle, it can be estimated that 20—50 acetylcholinesterase dimers are present per vesicle at a lipid-to-protein ratio of 20 : 1 (w/w) and 4—10 dimers at a lipid-to-protein ratio of 100 : 1 (w/w). The above-made considerations suggest that replacement of Triton X-100 by phosphatidylcholine during the reconstitution procedure preserves the enzyme largely in its dimeric state in spite of the fact that several acetylcholinesterase molecules are present in the membrane of one vesicle.

It is attractive to suggest that the same hydrophobic lipid-protein interactions which, in phospholipid vesicles, largely prevent the formation of acetylcholinesterase oligomers, preserve the dimeric species in the native membrane.

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