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A Small Hydrophobic Domain That Localizes Human Erythrocyte Acetylcholinesterase in Liposomal Membranes Is Cleaved by Papain Digestion†

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ABSTRACT: A small hydrophobic domain in isolated human erythrocyte acetylcholinesterase is responsible for the interaction of this enzyme with detergent micelles and the aggregation of the enzyme on removal of detergent. Papain has been shown to cleave this hydrophobic domain and to generate a fully active hydrophilic enzyme that shows no tendency to interact with detergents or to aggregate [Dutta-Choudhury, T. A., & Rosenberry, T. L. (1984) *J. Biol. Chem. 259*, 5653-5660]. We report here that the intact enzyme could be reconstituted into phospholipid liposomes while the papain-disaggregated enzyme showed no capacity for reconstitution. More than 80% of the enzyme reconstituted into small liposomes could be released by papain digestion as the hydrophilic form. Papain was less effective in releasing the enzyme from large liposomes that were probably multilamellar. In a novel application of affinity chromatography on acridinium resin, enzyme reconstituted into small liposomes in the presence of excess phospholipid was purified to a level of 1 enzyme molecule per 4000 phospholipid molecules, a ratio expected if each enzyme molecule was associated with a small, unilamellar liposome. Subunits in the hydrophilic enzyme form released from reconstituted liposomes by papain digestion showed a mass decrease of about 2 kilodaltons relative to the intact subunits according to acrylamide gel electrophoresis in sodium dodecyl sulfate, a difference similar to that observed previously following papain digestion of the soluble enzyme aggregates. The data were consistent with the hypothesis that the same hydrophobic domain in the enzyme is responsible for the interaction of the enzyme with detergent micelles, the aggregation of the enzyme in the absence of detergent, and the incorporation of the enzyme into reconstituted phospholipid membranes.

Acetylcholinesterase (AChE)[1] (EC 3.1.1.7) forms in vertebrate tissues are classified either as asymmetric if they include a collagen-like tail structure or as globular if they are devoid of such a structure [see Massoulié & Bon (1982) and Rosenberry (1985)]. In addition to soluble and secreted forms, the globular AChEs include a class of integral membrane

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[1] Abbreviations: AChE, acetylcholinesterase; RBC AChE, human erythrocyte acetylcholinesterase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; kDa, kilodalton(s).

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AChE forms with a hydrophobic domain that interacts with cell plasma membranes. These globular membrane-bound AChEs are the predominant AChE forms in mammalian brain and in muscle cells outside the neuromuscular junctions. A dimeric G_2 form[2] in this class is the only AChE present on human erythrocytes, and the availability of outdated human erythrocytes has permitted the study of human erythrocyte AChE (RBC AChE) as a general model for integral membrane AChE forms. These forms require nonionic detergents for extraction and continue to interact with detergents following their solubilization (Massoulié & Bon, 1982). Highly purified RBC AChE associates with detergent in micelles (Wiedmer et al., 1979; Rosenberry & Scoggin, 1984) but forms soluble aggregates on removal of detergent (Ott & Brodbeck, 1978). RBC AChE is an amphipathic protein because its small hydrophobic domain may be cleaved from the remaining hydrophilic enzyme by papain digestion of either enzyme–detergent micelles or soluble enzyme aggregates (Dutta-Choudhury & Rosenberry, 1984). The hydrophilic enzyme fragment has virtually the same polypeptide size as the intact amphipathic enzyme and retains full enzyme activity, but it no longer associates with detergents or forms aggregates in the absence of detergent.

Before proceeding with characterization of the hydrophobic domain cleaved by papain, it is important to confirm the presumption that the same hydrophobic domain which causes aggregation and detergent interaction in RBC AChE also localizes RBC AChE in phospholipid membranes. This point is particularly important because papain is relatively ineffective in releasing RBC AChE from intact human erythrocytes (Dutta-Choudhury & Rosenberry, 1984). In this paper, we demonstrate that papain digestion abolishes the capacity for RBC AChE to reconstitute into phospholipid liposomal membranes and releases RBC AChE from liposomes reconstituted with the intact enzyme by cleaving the same hydrophobic domain. This domain is of great interest because of its novel structure. In RBC AChE, it is composed of nonamino acid components that include glucosamine and ethanolamine (Haas et al., 1985; Rosenberry et al., 1985). In an amphipathic G_2 AChE from torpedo electric organ that is similar in many respects to RBC AChE, the hydrophobic domain is cleaved by a phosphatidylinositol-specific phospholipase C (Futerman et al., 1984). In addition, this paper demonstrates that liposomes reconstituted with RBC AChE can be purified by affinity chromatography to an extent that permits estimation of the size of the reconstituted liposomes.

Materials and Methods

Proteins. RBC AChE was extracted from outdated erythrocytes with Triton X-100, purified by affinity chromatography on acridinium resin, and depleted of Triton X-100 by chromatography on hydroxyapatite as described by Rosenberry & Scoggin (1984). G_4 AChE from the eel *Electrophorus electricus* was prepared by digesting purified 18S plus 14S eel AChE (0.5 mg/mL in 1.0 M NaCl, 5 mM decamethonium bromide, and 20 mM sodium phosphate, pH 7; Rosenberry et al., 1982) with trypsin (1 mg/mL) for 1 h at 25 °C and separating the AChE activity peak corresponding to a Stokes radius (R_s) of 8.8 nm on Sepharose CL-4B in 20 mM sodium phosphate, pH 7. Bovine catalase and *Escherichia coli* β-galactosidase (R_s = 5.2 and 8.2 nm, respectively; Bon et al., 1976) and aqueous suspensions of papain (25–28 mg/mL) were the best grade available from Sigma Chemical Co. Papain-linked Sepharose CL-4B (2.5 mg of papain per milliliter of packed resin; papain resin) was prepared as outlined in Dutta-Choudhury & Rosenberry (1984).

Analytical Procedures. AChE assays, AChE protein determination, PAGE in SDS, and active-site labeling with [^{3}H]DFP were conducted as indicated in Rosenberry & Scoggin (1984). Gel exclusion chromatography on a 120-mL Sepharose CL-4B column (1.5 × 70 cm) at 4 °C involved 0.5–4-mL sample onputs to which 20 μL of saturated $K_2Cr_2O_7$ had been added as a solvent marker. Phospholipid determinations as inorganic phosphate followed Ames (1966).

Reconstitution of RBC AChE Liposomes. Reconstitutions were based on the cholate dialysis procedure of Kagawa & Racker (1971). Reconstitution mixtures contained RBC AChE (20–170 μg/mL), crude egg yolk phospholipid (Sigma Chemical Co. type IX-E, approximately 60% phosphatidylcholine; 0.25–50 mg/mL), 1.4% sodium deoxycholate (Sigma Chemical Co.), 2 mM sodium azide, 1 mM edrophonium chloride, and 10 mM Tris-HCl, pH 7.4, and were dialyzed against 3 mM sodium azide, 30 μM edrophonium chloride, and 15 mM Tris-HCl, pH 7.4, for 24–72 h to form liposomes. The protein content of the phospholipid (estimated by amino acid analysis) was <0.05% prior to dialysis and <0.01% after dialysis. Reconstitution results were essentially the same for sodium deoxycholate and sodium cholate in preliminary experiments, but sodium deoxycholate was selected for further work to permit comparison to a previous report on RBC AChE (Hall & Brodbeck, 1978).

Stock phospholipid prior to addition to the reconstitution mixture was prepared in two ways: (1) Phospholipid suspensions in distilled water (20–150 mg/mL) were flushed briefly with argon and sonicated in a Branson B-220H bath-type sonicator for 30–90 min at 0–10 °C. The suspensions were centrifuged for 5 min in a table-top centrifuge, and aliquots of the opaque supernatants were added to the reconstitution mixture. (2) Phospholipid was dissolved in diethyl ether (8 mg/mL) and dried to a thin film on a rotary evaporator. Following addition of distilled water, an opaque phospholipid suspension (20–190 mg/mL) was generated by bath sonication as above for 30 min and clarifed by further sonication with a Heat Systems-Ultrasonics Model W200R probe-type sonicator with a standard microtip. The total probe sonication time was 2–30 min divided into 15–30–s bursts at 0–5 °C. Some reconstituted preparations were further treated by one or more freeze–thaw cycles involving freezing in a −20 °C freezer and thawing at ambient temperature (Anholt et al., 1982).

Papain Digestion of RBC AChE Reconstituted in Liposomes. Activation of papain resin by exposure to a thiol reducing agent followed by removal of the reducing agent and digestion of reconstituted RBC AChE was conducted as outlined in Dutta-Choudhury & Rosenberry (1984). Soluble papain was similarly activated by mixing with an equal volume of the reducing agent stock for 15 min at room temperature. The reducing agent was effectively diluted out in the digests by activation mixture dilutions of at least 50-fold. Papain digests included 0.1–1.0 mM edrophonium chloride to minimize RBC AChE degradation (Dutta-Choudhury & Rosenberry, 1984).

Results

Liposome preparations in this study were based on the cholate dialysis procedure of Kagawa & Racker (1971). Initial reconstitution mixtures included RBC AChE, egg phospholipid, and buffered 1.4% sodium deoxycholate and generally were dialyzed for 48 h at 4 °C, although longer dialysis ap-

[2] G_n refers to a globular AChE form with n catalytic subunits.

peared to have little effect on the hydrodynamic properties of the liposomes reconstituted with RBC AChE (RBC AChE liposomes). Preliminary experiments indicated that RBC AChE lost over 90% of its enzymatic activity under these reconstitution conditions.[3] Sodium deoxycholate has been reported to rapidly inactivate RBC AChE, and egg phosphatidylcholine only partially protected the enzyme against this inactivation (Hall & Brodbeck, 1978). The competitive inhibitor edrophonium chloride has been shown to protect several AChEs against inactivation by proteases or denaturants (Dutta-Choudhury & Rosenberry, 1984). Inclusion of this inhibitor at 1 mM in the initial reconstitution mixture and at 30 μM in the dialysis solution was sufficient to protect the enzyme activity. Only about 15% of the activity typically was lost under these conditions.

The size of the reconstituted RBC AChE liposomes was controlled both by the conditions under which the stock phospholipid was sonicated prior to reconstitution and by the use of a freeze–thaw cycle to increase the liposome size (Anholt et al., 1982) after reconstitution. In initial experiments, large liposomes were desired so that successful reconstitution of RBC AChE into liposomal membranes could be monitored easily by gel exclusion chromatography. Stock phospholipid was sonicated only with a bath sonicator, and the highly turbid reconstituted liposome mixture was frozen prior to chromatography. Results with liposomes reconstituted by this procedure are shown in Figure 1. In each case, the turbid liposome fraction was eluted at the void volume of a Sepharose CL-4B column. More vigorous sonication of the stock phospholipid with a probe-type sonicator yielded smaller reconstituted liposomes as described below.

The capacity of three different types of AChE for reconstitution into large liposomes was examined in Figure 1. Intact G_2 RBC AChE interacts through a small hydrophobic domain to form soluble aggregates that are characterized by a Stokes radius (R_s) of 10 nm (Rosenberry & Scoggin, 1984). These RBC AChE aggregates eluted at a partially included volume on a Sepharose CL-4B column prior to reconstitution (Figure 1A). When the aggregates were included in a reconstitution mixture, the RBC AChE was incorporated into large liposomes and eluted in the column void volume (Figure 1A). Papain digestion of intact RBC AChE removes the hydrophobic domain and generates a G_2 AChE dimer with a Stokes radius of 5.8 nm that no longer aggregates or interacts with detergents (Dutta-Choudhury & Rosenberry, 1984). To investigate whether this hydrophobic domain is responsible for the interaction of RBC AChE with phospholipid membranes, we first examined the capacity of the papain-disaggregated G_2 enzyme to reconstitute into liposomes. As shown in Figure 1B, inclusion of this G_2 enzyme in a reconstitution mixture had no effect on the enzyme elution volume. This result indicated that removal of the hydrophobic domain by papain also abolished the capacity of RBC AChE to interact with phospholipid liposomes. As an additional control, the reconstitution of eel G_4 AChE generated by trypsin digestion of A_{12} and A_8 AChE was investigated. Detergent binding (Millar et al., 1978) and hydrodynamic studies (Bon et al., 1978) indicate that this G_4 AChE contains no hydrophobic domain and, as anticipated, this AChE showed no evidence of reconstitution into liposomes (Figure 1C).

More vigorous sonication of the stock phospholipid prior to its addition to the reconstitution mixture resulted in the formation of small liposomes which were eluted from Sepharose

[3] A. Burke and T. L. Rosenberry, unpublished observations.

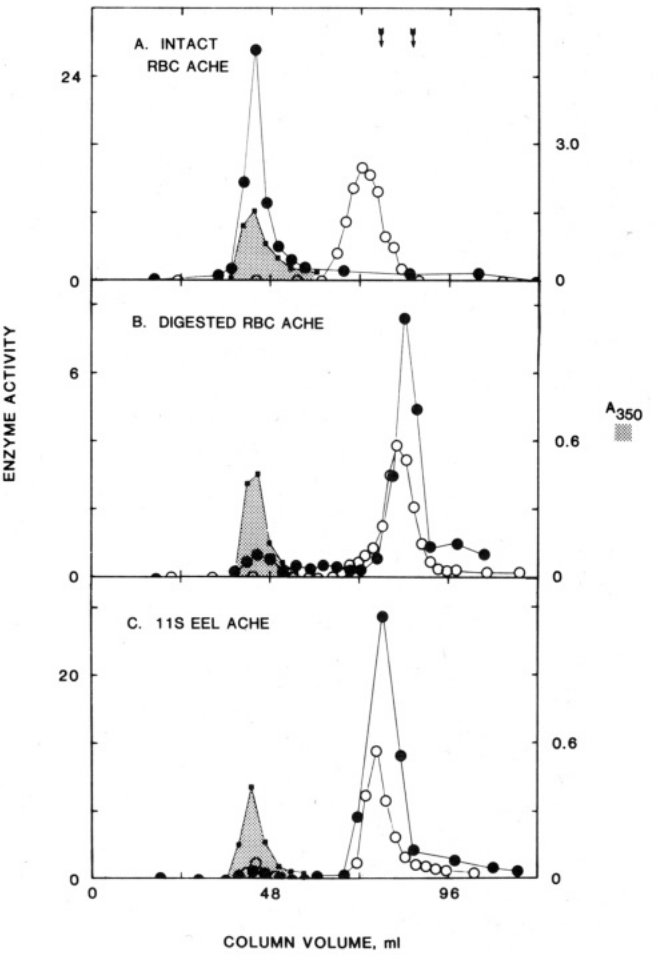

FIGURE 1: Liposome reconstitution tests for RBC and eel AChEs. Reconstitution mixtures (AChE, 25–100 units; bath-sonicated phospholipid, 15 mg in 1 mL) were prepared, taken through one freeze–thaw cycle, and chromatographed on Sepharose CL-4B in 20 mM sodium phosphate (pH 7.0) as outlined under Materials and Methods. (●) Enzyme activity (units) in reconstitution mixtures; (○) enzyme activity (units) in AChE control samples chromatographed prior to reconstitution; (shaded areas) turbidity (A_{350nm}). The elution positions of standards are indicated by arrows (from left, β-galactosidase and catalase). The elution volume of the solvent marker $K_2Cr_2O_7$ corresponds to the right axis. (A) Intact RBC AChE. (B) Disaggregated RBC AChE prepared from intact RBC AChE by papain digestion and isolated by Sepharose CL-4B chromatography (Dutta-Choudhury & Rosenberry, 1984) prior to reconstitution. Activity units of the control disaggregated RBC AChE are scaled to one-sixth of the actual values. (C) Trypsin-digested G_4 eel AChE.

CL-4B at a partially included volume. The profile in Figure 2A indicated a preparation that contained a mixture of large and small RBC AChE liposomes, but preparations that were almost exclusively small liposomes were frequently obtained. The relative elution volume of the small RBC AChE liposomes varied slightly from preparation to preparation but was always less than that of the smaller soluble AChE aggregates. This point was demonstrated in Figure 2B, where a selected fraction of small RBC AChE liposomes was rechromatographed with aggregated [^{3}H]DFP-labeled RBC AChE. The elution volume of the RBC AChE liposomes was unchanged and clearly distinct from that of the labeled RBC AChE, which corresponded precisely to that previously observed for unlabeled RBC AChE aggregates (Rosenberry & Scoggin, 1984). Figure 2B also demonstrated that aggregated RBC AChE did not interact with preformed liposomes. A similar lack of interaction between preformed [^{3}H]DFP-labeled RBC AChE liposomes and active aggregated RBC AChE was observed even when the mixture was incubated for 48 h at 4 °C (data

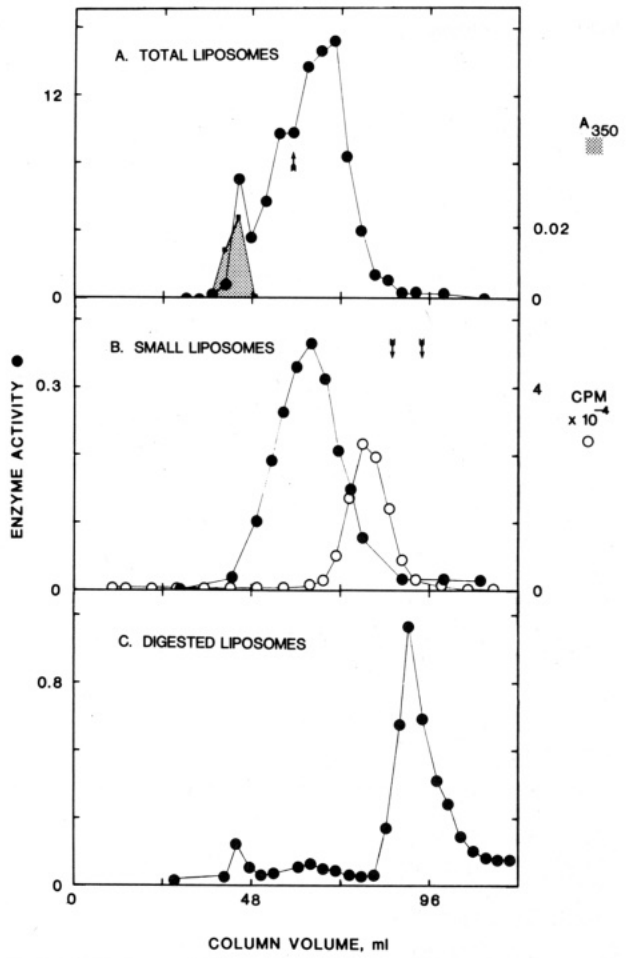

FIGURE 2: Release of RBC AChE from small liposomes by papain. Samples were analyzed by chromatography on Sepharose CL-4B in 20 mM Tris-HCl (pH 7.4) as outlined under Materials and Methods. The reconstitution mixture (1.0 mL) included 200 units of RBC AChE and 2 mg of phospholipid (the stock phospholipid had been briefly probe-sonicated for 2 min) and was dialyzed as indicated under Materials and Methods. (●) Enzyme activity (units). The arrows in panel B indicate the elution positions of the same standards used in Figure 1. (A) Elution profile from the entire reconstitution mixture; turbidity (A_{350nm}) is shaded. (B) Rechromatography of a small liposome fraction. A 1.0-mL aliquot of the fraction indicated by the arrow in (A) was mixed with 30 μL of [^{3}H]DFP-labeled control RBC AChE. (○) ^{3}H cpm. (C) A 2.0-mL aliquot of the fraction indicated by the arrow in (A) (5 units) was mixed with activated soluble papain (0.14 mg), incubated for 1 h at 25 °C, and chromatographed. Recovery of enzyme activity from the column in (C) was 70% of the activity originally digested.

not shown). This result is consistent with the failure of RBC AChE aggregates to interact with a preformed lipid bilayer (Wiedmer et al., 1978). Exposure of purified RBC AChE aggregates to intact human erythrocytes also resulted in no association of the purified enzyme activity with the erythrocytes.[3]

As a second test of the hypothesis that the hydrophobic domain removed by papain digestion is responsible for the interaction of RBC AChE with phospholipid membranes, we investigated directly the effects of papain on RBC AChE liposomes. Small RBC AChE liposomes were incubated with papain at 25 °C for 1 h in the presence of 0.1 mM edrophonium chloride to prevent papain inactivation of the RBC AChE (Dutta-Choudhury & Rosenberry, 1984). The digest was applied to Sepharose CL-4B, and the profile in Figure 2C was obtained. More than 80% of the RBC AChE activity had been released from the liposomes and was eluted precisely at the position of the papain-disaggregated RBC AChE obtained

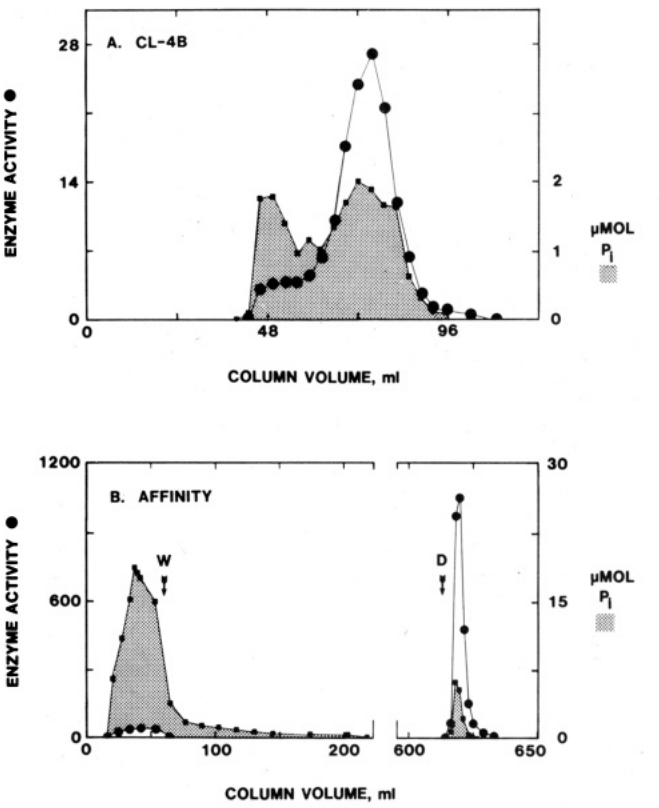

FIGURE 3: Large-scale preparation of small RBC AChE liposomes and their purification by affinity chromatography. The reconstitution mixture (550 units/mL; probe-sonicated phospholipid, 50 mg/mL; 16.5 mL) was prepared and dialyzed as outlined under Materials and Methods. (●) Enzyme activity (units). Phospholipid determined as micromoles of inorganic phosphate (shaded). (A) An aliquot (140 units) of the dialyzed reconstitution mixture was chromatographed on Sepharose CL-4B under the conditions in Figure 2. (B) The remainder of the dialyzed reconstitution mixture was applied to a 25-mL column of acridinium affinity resin (Rosenberry & Scoggin, 1984) equilibrated in 15 mM Tris-HCl (pH 7.5). Following introduction of the sample, the column was washed (W) with 300 mL of the equilibration buffer at a rate of 0.2 column volume/h, and RBC AChE liposomes were eluted with 10 mM decamethonium bromide (D) in 10 mM Tris-HCl (pH 7.5). In (B), enzyme activity is in units per milliliter and inorganic phosphate in micromoles per milliliter.

from digests of RBC AChE aggregates (see Figure 1B). Thus, the site of papain cleavage that releases the hydrophobic domain from the active hydrophilic domain of RBC AChE is accessible to papain in these RBC AChE liposomes.

Larger quantities of RBC AChE liposomes were required to characterize the site of papain cleavage, and reconstitution was routinely carried out in the presence of excess phospholipid under conditions that produced predominantly small RBC AChE liposomes. A typical preparation is shown in the Sepharose CL-4B profile in Figure 3A. Most of the RBC AChE activity corresponded to small RBC AChE liposomes, but bulk phospholipid was about equally divided between large and small liposomes according to inorganic phosphate determinations. Electron microscopy revealed a relatively homogeneous population of vesicular structures in fractions corresponding either to large or to small liposomes, with average diameters of 75 and 22 nm, respectively.[4] RBC AChE liposomes could be separated from bulk liposomes by affinity chromatography on acridinium resin, the same resin used to purify detergent-solubilized RBC AChE. As shown in Figure

[4] H. Eichelberger, unpublished observations. Electron microscopy was conducted on samples applied to 500-mesh copper grids with a carbon support film and negative stained with ammonium molybdate.

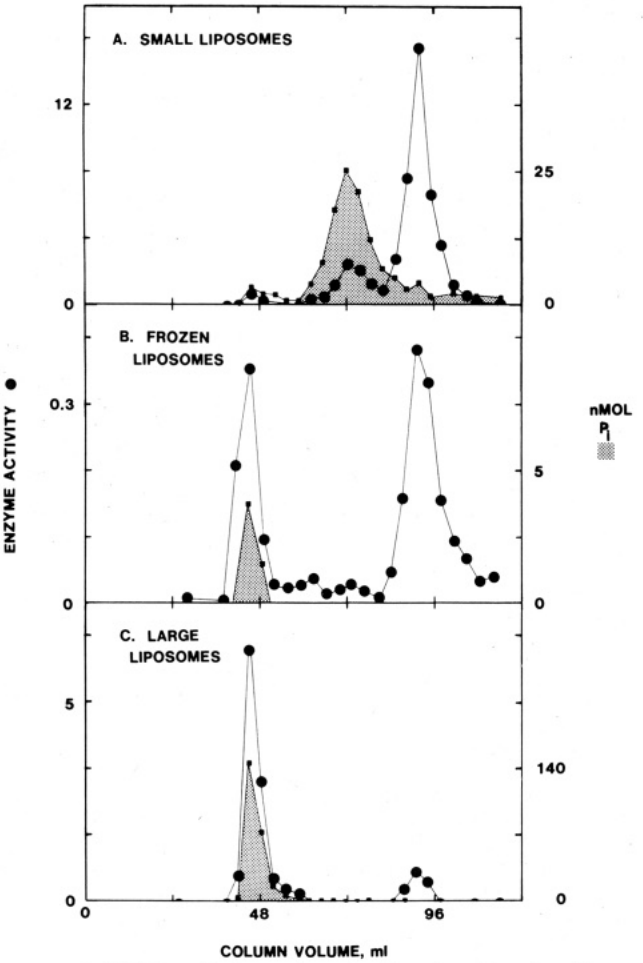

FIGURE 4: Relative efficiency of papain digestion of small and large RBC AChE liposomes. Chromatography conditions on Sepharose CL-4B were the same as those in Figure 2. The retained RBC AChE liposome pool from Figure 3B was dialyzed overnight to remove decamethonium bromide. Sepharose CL-4B chromatography of two aliquots (2 × 4 mL) of the dialyzed pool indicated 11% large RBC AChE liposomes and 88% small RBC AChE liposomes (data not shown). A third aliquot (4 mL) of the dialyzed pool was taken through two cycles of overnight freezing at −20 °C with a 2-h thaw between cycles. Chromatography of this freeze–thaw aliquot on Sepharose CL-4B indicated 46% large RBC AChE liposomes and 52% small RBC AChE liposomes (data not shown). Activated soluble papain (0.5 mg) was added to selected Sepharose CL-4B column fractions (50–200 enzyme units in 3 mL) containing 1 mM edrophonium chloride at 25 °C (see Materials and Methods). A second addition of papain (0.5 mg) was made after 1 h, and 1 h later the mixture was applied to Sepharose CL-4B. (A) Small RBC AChE liposome digest. (B) Digest of large RBC AChE liposomes prepared by freeze–thaw cycles. (C) Digest of large RBC AChE liposomes generated in the original sonication and reconstitution. Enzyme activity (●) is in units, and phospholipid (shaded) is in nanomoles of inorganic phosphate. Recoveries of enzyme activity from the columns (10–25% of the activities originally digested with papain) were lower here than in Figure 2C, perhaps because these highly purified RBC AChE liposomes were more susceptible to papain inactivation or to losses by adsorption.

3B, bulk liposomes together with some RBC AChE liposomes passed directly through the affinity column in a nonretained pool, although extensive column washing was required to remove residual bulk liposomes. Subsequent elution of the retained RBC AChE liposomes with the eluting ligand decamethonium bromide gave peak fractions with a nearly constant ratio of enzyme activity to phospholipid that corresponded to about 0.25 nmol of enzyme dimer per micromole of inorganic phosphate. The total recovery of RBC AChE liposomes from the affinity column was typically quite high (>95%), but the

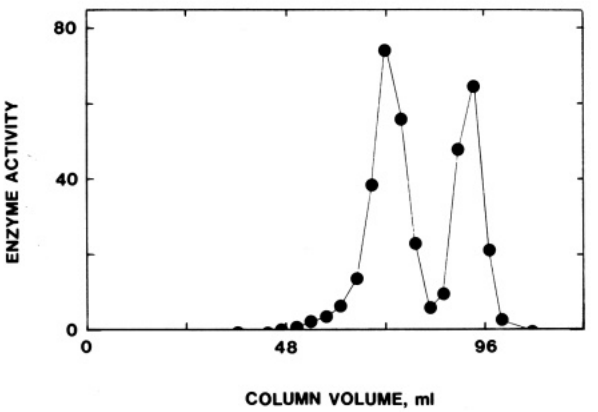

FIGURE 5: Digestion of small RBC AChE liposomes with papain resin and fractionation of the digest on Sepharose CL-4B. Chromatography conditions were the same as in Figure 2. Following reconstitution (RBC AChE, 300 units/mL; probe-sonicated phospholipid, 50 mg/mL), affinity chromatography (as in Figure 3B), and dialysis, RBC AChE liposomes (1100 units in 8 mL) were digested with activated papain resin (2 mL) at 4 °C as outlined under Materials and Methods. The digest supernatant was subjected to affinity chromatography, and retained enzyme corresponding to residual RBC AChE liposomes and papain-released G_2 enzyme was eluted with decamethonium bromide, dialyzed, and applied to Sepharose CL-4B. The elution volume of the first peak of enzyme activity corresponds to residual small RBC AChE liposomes while that of the second peak coincides precisely with papain-disaggregated G_2 RBC AChE in Figure 2. Enzyme activity is in units. Recovery of enzyme activity corresponded to 55% of the activity digested.

distribution of AChE activity between nonretained liposomes and retained, purified RBC AChE liposomes varied among preparations. Systematic investigation of this point revealed that nonretained RBC AChE liposomes invariably were entirely large, while the retained RBC AChE liposomes were predominantly small (data not shown).

Large and small RBC AChE liposomes also differed in their susceptibility to papain digestion. RBC AChE liposomes prepared by sonication and purified by affinity chromatography were fractionated into large and small categories by chromatography on Sepharose CL-4B. A portion of the small RBC AChE liposome pool was taken through two freeze–thaw cycles, and the large RBC AChE liposomes generated by these cycles were isolated by chromatography on Sepharose CL-4B. Papain digests of the small RBC AChE liposomes and the two classes of large RBC AChE liposomes were analyzed on Sepharose CL-4B in Figure 4. About 80% of the RBC AChE activity was released from the small liposomes by papain digestion and appeared at the elution volume characteristic of the papain-disaggregated G_2 RBC AChE (Figure 4A). In contrast, only about 55% of the enzyme activity was released from the large RBC AChE liposomes prepared by the freeze–thaw cycles (Figure 4B), and only 13% of the activity was released from the large RBC AChE liposomes generated by the original sonication and reconstitution procedure (Figure 4C).

To assess the site of papain cleavage which releases enzyme activity from RBC AChE liposomes, we characterized intact and papain-released enzyme by SDS–PAGE. Residual soluble papain rapidly degrades RBC AChE samples exposed to SDS, but this degradation can be avoided if digestion is conducted with papain resin (Dutta-Choudhury & Rosenberry, 1984). RBC AChE liposomes were purified by affinity chromatography and digested with papain resin under conditions that result in partial release of RBC AChE. Released and liposomal RBC AChEs in the digest supernatant were subjected to further affinity chromatography as outlined in the Figure

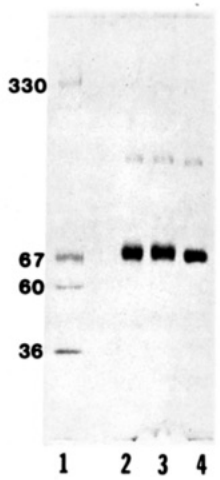

FIGURE 6: Analysis of catalytic subunits in RBC AChE liposomes and papain-released G_2 RBC AChE by SDS–PAGE. The gradient slab gel was prepared, run, and stained with silver as outlined by Rosenberry & Scoggin (1984). Lane 1, polypeptide standards (Pharmacia Fine Chemicals) with their molecular masses indicated in kilodaltons (from top): thyroglobulin, bovine serum albumin, catalase, and lactate dehydrogenase. Lanes 2–4, 0.5 μg of RBC AChE samples: (lane 2) control purified enzyme; (lane 3) enzyme from small RBC AChE liposomes in the first peak in Figure 5; (lane 4) papain-released G_2 enzyme from the second peak in Figure 5.

5 legend to remove any inactive enzyme fragments generated by the papain digestion. The distribution of RBC AChE liposomes and released G_2 RBC AChE was determined on Sepharose CL-4B in Figure 5, and samples of each component were analyzed by SDS–PAGE in Figure 6. The apparent molecular mass of the fully reduced control RBC AChE subunits in lane 2 of Figure 6 is consistent with the previous report of 75 kDa [see Rosenberry & Scoggin (1984) and Dutta-Choudhury & Rosenberry (1984)]. Lane 3 of Figure 6 indicates that no change in the apparent mass occurs with the subunits from RBC AChE liposomes in Figure 5, but the G_2 RBC AChE released from the liposomes by papain shows a catalytic subunit mass decrease of about 2 kDa in lane 4 of Figure 6. A similar relationship was observed by SDS–PAGE for these three RBC AChE samples prior to reduction of their intersubunit disulfide bonds (data not shown). The banding pattern was identical with that observed in lanes 2–4 for the fainter, residual subunit dimer bands. This apparent decrease in subunit mass on papain cleavage and release of RBC AChE from liposomes is identical with the mass decrease observed when papain cleaved the purified RBC AChE aggregates to disaggregated G_2 (Dutta-Choudhury & Rosenberry, 1984). The identity of the hydrophobic domains responsible for RBC AChE aggregation, detergent micelle formation, and incorporation into liposomes is considered further under Discussion.

Discussion

The capacity of purified RBC AChE for reconstitution into liposomal membranes by a variety of detergent depletion methods was demonstrated by Brodbeck and his colleagues (Hall & Brodbeck, 1978; Römer-Lüthi et al., 1980). The loss of this capacity by RBC AChE which had been disaggregated by papain digestion of purified RBC AChE (Figure 1) and the generation of a hydrophilic G_2 RBC AChE by papain digestion of small RBC AChE liposomes (Figure 2) indicate strongly that the same hydrophobic domain is removed by both digestion conditions. More quantitative evidence of the similarity of the hydrophobic domains removed by these digestions is shown in Figure 6, where the decrease in subunit mass produced by papain digestion of RBC AChE liposomes is the same as that observed previously for papain digestion of RBC AChE aggregates (Dutta-Choudhury & Rosenberry, 1984). Finally, a direct comparison of the hydrophobic domains cleaved by the two digestion conditions has been conducted following radiolabeling with the probe [^{125}I]TID. This reagent partitions selectively into the hydrophobic phase of membranes, liposomes, and protein–detergent micelle complexes and, on photoactivation, covalently reacts with molecules in the hydrophobic phase (Brunner & Semenza, 1981). Following [^{125}I]TID labeling and repurification of the labeled intact RBC AChE, papain digestion of either RBC AChE aggregates or RBC AChE liposomes demonstrated the exclusive localization of the [^{125}I]TID label in the released hydrophobic domain (Dutta-Choudhury & Rosenberry, 1984; Roberts & Rosenberry, 1985).[5] Furthermore, the sizes of the labeled hydrophobic domain released by either digestion procedure were identical according to gel exclusion chromatography in organic solvents on Sephadex LH-60. Thus, we conclude that the same hydrophobic domain in RBC AChE is responsible for the formation of enzyme–detergent micelles, aggregation in the absence of detergents, and incorporation into liposomes.

The demonstration of release of enzyme from RBC AChE liposomes on digestion with papain depended on several technical considerations in the preparation of the RBC AChE liposomes. When purified egg phosphatidylcholine (Sigma type VII-E) was substituted for the usual crude egg phospholipid in reconstitution tests which paralleled that in Figure 1A, enzyme activity and turbidity cochromatographed near the solvent volume indicated by $K_2Cr_2O_7$. This observation suggested an interaction between Sepharose CL-4B and liposomes prepared with purified phosphatidylcholine under our chromatography conditions. Because this late elution could interfere with an interpretation of AChE release following papain digestion, purified phosphatidylcholine was abandoned, and all further experiments were carried out with crude egg phospholipid. The size of the RBC AChE liposomes was also an important consideration in determining the extent of release during papain digestion, as indicated in Figure 4. RBC AChE liposome size in the initial reconstitution mixture is determined by numerous factors including the method of detergent depletion (Hall & Brodbeck, 1978; Römer-Lüthi et al., 1980), the dialysis time (when detergent is depleted by dialysis), and, in our hands, the extent of sonication of the phospholipid stock. Large liposomes frequently are multilamellar (Bangham et al., 1965), and this could account both for their lower retention on affinity resins (see Results) and for their lower susceptibility to RBC AChE release by papain (Figure 4C). Proteases in general inactivate a smaller percentage of AChE activity with large RBC AChE liposomes than with small RBC AChE liposomes (Hall & Brodbeck, 1978; Römer-Lüthi et al., 1980), consistent with this suggestion. The enzyme in small RBC AChE liposomes appeared almost completely accessible to papain (Figures 2C and 4A), an indication of a nearly exclusive external orientation of the RBC AChE in these liposomes [also see Hall & Brodbeck (1978)]. Large RBC AChE liposomes formed by freezing and thawing small RBC AChE liposomes showed partial release of AChE activity by papain (Figure 4B) and thus would appear to be less multilamellar than the large liposomes in the initial reconstitution mixtures.

Affinity chromatography of small RBC AChE liposomes on acridinium resins permitted the purification of more than 80% of the applied enzyme activity from bulk liposomes in the

[5] Similar results with [^{125}I]TID have been obtained with an amphipathic AChE from torpedo electric organ (Stieger et al., 1984).

reconstitution mixture. This technique for the purification of a liposomal protein from the reconstitution mixture, to our knowledge, has not been reported previously. The number of phospholipid molecules per G_2 RBC AChE molecule in the purified fractions was about 4000, and this number corresponds to a small unilamellar liposome 24 nm in diameter [assuming a 5-nm bilayer thickness and 0.6-nm^2 surface area per phospholipid molecule (Shah & Schulman, 1967)]. This liposome size estimate is very close to the average size of 22 nm for small liposomes measured by electron microscopy as noted under Results, and it agrees with a previous report of 21 nm for homogeneous small unilamellar liposomes (Barenholz et al., 1977). Thus, the purified RBC AChE liposome pool appears to consist of small liposomes with one enzyme molecule per liposome.

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