Physicochemical behaviour and structural characteristics of membrane-bound acetylcholinesterase from *Torpedo* electric organ

Effect of phosphatidylinositol-specific phospholipase C

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Quantitative solubilization of the phospholipid-associated form of acetylcholinesterase (AChE) from *Torpedo* electric organ can be achieved in the absence of detergent by treatment with phosphatidylinositol-specific phospholipase C (PIPLC) from *Staphylococcus aureus* [Futerman, Low & Silman (1983) *Neurosci. Lett.* **40**, 85–89]. The sedimentation coefficient on sucrose gradients of AChE solubilized in detergents (DSACHe) varies with the detergent employed. However, the sedimentation coefficient of AChE directly solubilized by PIPLC is not changed by detergents. Furthermore, PIPLC can abolish the detergent-sensitivity of the sedimentation coefficient of DSACHe purified by affinity chromatography, suggesting that one or more molecules of phosphatidylinositol (PI) are co-solubilized with DSACHe and remain attached throughout purification. DSACHe binds to phospholipid liposomes, whereas PIPLC-solubilized AChE and DSACHe treated with PIPLC do not bind even to liposomes containing PI. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis shows that PIPLC-solubilized AChE, like unmodified DSACHe, is a catalytic subunit dimer; electrophoresis in the presence of reducing agent reveals no detectable difference in the M_r of the catalytic subunit of unmodified DSACHe, of AChE solubilized by PIPLC and of AChE solubilized by Proteinase K. The results presented suggest that DSACHe is anchored to the plasma membrane by one or more PI molecules which are tightly attached to a short amino acid sequence at one end of the catalytic subunit polypeptide.

A substantial part of the acetylcholinesterase (AChE; acetylcholine hydrolase, EC 3.1.1.7) in *Torpedo* electric organ occurs as a hydrophobic catalytic subunit dimer (Bon & Massoulié, 1980; Viratelle & Bernhard, 1980; Witzemann, 1980) that displays the solubility characteristics of an integral membrane protein inasmuch as it is only

soluble in the presence of detergent (DSACHe). However, we have recently shown that this hydrophobic form of AChE is selectively solubilized, in the absence of detergent, both from the particulate fraction and from a purified synaptosomal preparation of *Torpedo* electric organ by a phosphatidylinositol-specific phospholipase C (PIPLC; monophosphatidylinositol phosphodiesterase, EC 3.1.4.10) from *Staphylococcus aureus* (Futerman *et al.*, 1983, 1985). This raises the possibility that the catalytic subunit of AChE is anchored to the membrane via a specific interaction with the head-group(s) of one or more PI molecules. Here we present evidence based on physicochemical and enzymic studies to support the above suggestion that PI (or a derivative thereof) is indeed tightly associated with a small

Abbreviations used: AChE, acetylcholinesterase; DSACHe, detergent-soluble acetylcholinesterase; PIPLC, phosphatidylinositol-specific phospholipase C; PI, phosphatidylinositol; PC, phosphatidylcholine; PS, phosphatidylserine; DFP, di-isopropyl phosphorofluoridate; SDS, sodium dodecyl sulphate.

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sequence of the catalytic polypeptide of the DSAChe molecule. Some of these results have been reported in preliminary form (Futerman *et al.*, 1984).

Experimental

Materials

Torpedo californica electric organ was obtained from Pacific Biomarine (Venice, CA, U.S.A.) and stored at -80°C . PIPLC was purified from *Staphylococcus aureus* as previously described (Low, 1981). Phospholipase A_2 from bee venom, phospholipase A_2 from cobra (*Naja naja*) venom, phospholipase C (Type I) from *Clostridium welchii* and phospholipase D (Type IV) from cabbage (*Brassica oleracea*) were from Sigma. All these phospholipases were shown to be free of any proteolytic activity (Futerman *et al.*, 1985). Cholic acid (Merck) was recrystallized from hot ethanol. Brij 96, Triton X-100, Proteinase K (proteinase type XI from *Tritirachium album*), Pronase E (proteinase Type XIV from *Streptomyces griseus*), proteinase type XVII from *Staphylococcus aureus* (Strain V8) and papain (Type III, twice-crystallized) were all from Sigma. Thermolysin (proteinase type X) from Sigma, recrystallized as described by Blumberg & Tauber (1983), was a gift from Dr. S. Blumberg, Department of Biophysics, Weizmann Institute of Science, Rehovot 76-100, Israel. Bovine α -chymotrypsin (CDI purity) and bovine trypsin (TR purity) were from Worthington. Ox liver catalase, horse liver alcohol dehydrogenase and phenylmethanesulphonyl fluoride were from Sigma. Decamethonium bromide was from K and K (Plainview, NY, U.S.A.). [^3H]DFP (5.2 Ci/mmol) and [^3H]acetylcholine iodide (90 mCi/mmol) were from New England Nuclear Corp., Boston, MA, U.S.A.; the [^{14}C]methylated protein mixture and the NCS tissue solubilizer were from The Radiochemical Centre, Amersham, Bucks., U.K. Phospholipids (grade I) were obtained from Lipid Products, Redhill, Surrey, U.K. The affinity ligand [*N*-(6'-aminohexanoyl-6-aminohexanoyl)-*m*-aminophenyl]trimethylammonium bromide hydrobromide was a gift from Professor M. Liefänder and Dr. K. P. Ruess (University of Regensburg, Regensburg, Germany). Sucrose, buffers and salts were of analytical grade.

Purification of AChE from *Torpedo electric organ*

DSAChe was purified, after solubilization in sodium cholate, by a modification of the procedure of Viratelle & Bernhard (1980). Briefly, *Torpedo californica* electric organ (100-150 g) was chopped into small cubes and homogenized (1:3, w/v) in

buffer I (40 mM-MgCl₂/100 mM-NaCl/10 mM-Tris, pH 8.0) in a Sorvall Omnimixer (maximum speed, $2 \times 90\text{ s}$ at 4°C). The homogenate was centrifuged in a Sorvall GSA rotor for 1 h at 28000g and 4°C . The supernatant, containing about 10% of the total AChE activity, was the source of low-salt-soluble AChE. The pellet was rehomogenized in half the original volume, but with 2 M-MgCl₂/100 mM-NaCl/10 mM-Tris, pH 8.0. The resulting homogenate was diluted with buffer I before centrifugation in a 35Ti rotor using a Beckman L8-70 ultracentrifuge (78000g, 1 h, 4°C). This supernatant, containing 20-30% of the total AChE activity, was the source of high-salt-soluble AChE. The resulting pellet was rehomogenized in the original volume of buffer I, containing 1% sodium cholate, left for 1 h at 4°C , and centrifuged as described above (1 h, 78000g, 4°C). The final supernatant was passed over an affinity-chromatography column that was obtained by activation of Sepharose 2B (Pharmacia) with CNBr (Parikh *et al.*, 1974) and subsequent coupling of the (*m*-aminophenyl)trimethylammonium affinity ligand. The concentration of bound affinity ligand was 1-2.5 $\mu\text{mol/g}$ of wet resin. After application of the enzyme solution (450 ml), the affinity column (10 ml volume) was extensively washed with buffer I (500 ml) containing 1% sodium cholate. AChE was specifically eluted with either 1 mM- or 2 mM-decamethonium bromide in the same buffer. In some cases the eluted enzyme was dialysed against 3×1 litre of buffer I containing 1% sodium cholate, so as to remove the decamethonium, and then repurified by affinity chromatography as above.

AChE was also purified in the absence of detergent, subsequent to solubilization with Proteinase K, as follows. The pellet obtained after high-salt extraction was rehomogenized in the high-salt buffer so as to ensure complete removal of high-salt-soluble AChE; after re-centrifugation, the resulting pellet was rehomogenized in the original volume of buffer I and incubated with Proteinase K (100 $\mu\text{g/ml}$, 22°C). After 1 h, phenylmethanesulphonyl fluoride (1 mM) was added and the homogenate centrifuged at 78000g and 4°C for 1 h. Essentially all the remaining AChE was solubilized by this treatment. The Proteinase K-solubilized AChE was purified on an affinity column similar to that described for cholate-solubilized AChE, but all steps were carried out in buffer I without cholate.

The enzyme purified after solubilization with cholate or Proteinase K had a specific activity greater than 2500 units/mg (with 3 mM-[^3H]acetylcholine as substrate at pH 7.4), and appeared as one major band on SDS/polyacrylamide-gel electrophoresis.

Sucrose-gradient centrifugation

Analytical sucrose-gradient centrifugation was performed as previously described (Lyles *et al.*, 1979), with 5–20% (w/v) sucrose gradients in buffer I. When centrifugation was performed in the presence of detergent, the appropriate detergent was added to buffer I. Apparent sedimentation coefficients were determined by comparison with the standard marker enzymes catalase (11.4S) and alcohol dehydrogenase (5S).

Binding of AChE to liposomes

In order to investigate binding of AChE to artificial liposomes, a flotation gradient technique was used (Kaufmann & Silman, 1980). Phospholipids (4mg), dissolved in chloroform/methanol (2:1, v/v) were dried under N₂ and resuspended in 1 ml of 0.1 M-NaCl/0.1 M-phosphate, pH 7.0. The suspension was sonicated for 8 min at 25°C in a bath sonifier (Laboratory Supplies, Hicksville, NY, U.S.A.; model G112SPIT, 600 V). Samples of AChE (3 µl, containing 10–100 µg of purified enzyme/ml) were mixed with 280 µl of sonicated liposomes; the mixture was adjusted to a final volume of 0.7 ml by addition of 2 M-sucrose in 0.1 M-NaCl/0.1 M-phosphate, pH 7.0, to yield a final sucrose concentration of 1.2 M. The liposomes, in 1.2 M-sucrose, were layered over 0.5 ml of 2 M-sucrose. A 3.1 ml portion of 1 M-sucrose in the same buffer, followed by 0.5 ml of the buffer alone, were successively layered over the liposomes. The step gradients were then centrifuged in an SW50.1 rotor (50000 rev./min, 7 h, 4°C) using a Beckman L8-70 ultracentrifuge. After centrifugation, 0.2 ml fractions were collected and assayed for AChE activity. In all cases, the liposomes moved to the top of the gradient as monitored by measuring turbidity at 300 nm.

SDS/polyacrylamide-gel electrophoresis

Gels were prepared as described by O'Farrell (1975), 5–15% (w/v) gradient gels being routinely employed. Samples were boiled (5 min) in sample buffer [10% (v/v) glycerol/3% (w/v) SDS/0.62 M-Tris, pH 6.8] with or without β-mercaptoethanol (5%, v/v). In some cases, samples of AChE (50 µl) were labelled with [³H]DFP (10 µM) for 1 h at 22°C (Dudai & Silman, 1971) and co-electrophoresed with a mixture of [¹⁴C]methylated proteins (5 µCi/ml), which served as internal standards. Gels 20 cm long were used and cut into 1 mm slices. Slices were incubated for 4–5 h at 37°C in Xylofluor/xylene scintillation fluid containing 10% (v/v) NCS tissue solubilizer. Radioactivity was counted in a Packard Tri-Carb 4660 scintillation counter using a double-labelling program to process the data. The *M_r* value of [³H]DFP labelled AChE was calculated by comparing the electrophoretic mobility of [³H]DFP labelled AChE with that of five marker proteins, including ¹⁴C-bovine serum albumin.

AChE activity assay

AChE activity was assayed by the radiometric assay method of Johnson & Russell (1975), with [³H]acetylcholine as substrate.

Results

The apparent sedimentation coefficient (*s*) of DSACHe solubilized by PIPLC in the absence of detergent is 7.2S (the present Table 1; Futerman *et al.*, 1983). Addition of various detergents to the PIPLC-solubilized AChE has no influence on its sedimentation coefficient. For instance, AChE solubilized with Brij 96 or Triton X-100 has sedimentation coefficients of 5.0S and 6.3S respec-

Table 1. *Apparent sedimentation coefficient of Torpedo DSACHe in the presence of various detergents*
DSACHe was solubilized with either the designated detergent (1%, v/v) or by digestion with Proteinase K (100 µg/ml, 30 or 60 min, 22°C) or PIPLC (5 or 10 µg/ml, 37°C, 30 min) as indicated in the left-hand column. In one case (*), Brij 96-solubilized enzyme was subsequently treated with Proteinase K (100 µg/ml, 30 min, 22°C). Centrifugation was carried out on continuous 5–20% sucrose gradients either in buffer I alone or in buffer I containing the detergent designated at the top of the appropriate column. In cases where the detergent used during centrifugation differed from that used for solubilization, samples were diluted 100-fold into the new detergent and, after 1 h at 22°C, layered on to the gradients. Values of sedimentation coefficients represent means ± S.E.M., with the number of experiments shown in parentheses.

Mode of solubilization	Apparent sedimentation coefficient, <i>s</i> (S)			
	Buffer I	Brij 96	Triton X-100	Cholate
Brij 96	–	5.0 ± 0.5 (8)	–	7.3
Triton X-100	–	–	6.3 ± 0.4 (8)	–
Cholate	–	5.3 ± 0.2 (6)	–	7.3 ± 0.2 (7)
Brij 96/Proteinase K*	–	7.1 ± 0.4 (3)	–	–
Proteinase K	7.2 ± 0.2 (6)	7.0	6.8	–
PIPLC	7.2 ± 0.3 (10)	7.4 ± 0.1 (3)	7.1 ± 0.2 (4)	7.0

tively (Table 1), in agreement with previous reports (Bon & Massoulié, 1980; Viratelle & Bernhard, 1980), whereas the sedimentation coefficient of PIPLC-solubilized AChE remains at about 7.2S, even in the presence of either of these detergents. The effect of PIPLC thus appears similar, in physicochemical terms, to that of Proteinase K, which was reported previously (Bon & Massoulié, 1980) to render DSACHe insensitive to detergent by this criterion, a finding that we have confirmed (Table 1).

The sedimentation coefficient of DSACHe purified after cholate solubilization is 7.3S (Table 1); if this enzyme is diluted 100-fold into Brij 96, the sedimentation coefficient decreases to 5.3S, i.e. to a value similar to that obtained by direct solubilization with Brij 96 (Table 1). However, if incubation of the cholate-purified DSACHe with either PIPLC or Proteinase K precedes dilution of the enzyme into Brij 96, no such decrease is observed (Table 2). PIPLC was unable to modify directly the sedimentation coefficient of DSACHe solubilized in Brij 96, although Proteinase K could do so (Table 1). The failure of PIPLC to act in the presence of Brij 96 may be due to the fact that it is inhibited by non-ionic long-chain detergents (M. G. Low, unpublished work).

None of the other phospholipases tested, including a non-specific phospholipase C from *Clostridium welchii*, could prevent the decrease in sedi-

mentation coefficient that occurs on dilution of cholate-purified AChE into Brij 96 (Table 2). Furthermore, whereas Pronase E (a crude mixture of proteinases from *Streptomyces griseus*) acts similarly to Proteinase K in this respect (Bon & Massoulié, 1980), five other proteinases tested fail to have any significant effect (Table 2). Likewise, among these proteinases, Proteinase K and Pronase E were the only ones effective in solubilizing *Torpedo* DSACHe (Fig. 1), and PIPLC was the only phospholipase of those tested that was effective in solubilizing DSACHe from *Torpedo* electric organ and synaptosomal membranes (Futerman *et al.*, 1984).

Interaction of DSACHe with liposomes

By using the flotation-gradient technique, liposomes (and adhering AChE) float to the top of the gradient (Fig. 2b), whereas unbound AChE remains at the bottom (Fig. 2a). DSACHe, purified after cholate solubilization, binds almost completely to liposomes composed of a PC/PI mixture (Fig. 2b), whereas it fails to interact after prior treatment with PIPLC (Fig. 2d). AChE purified after Proteinase K solubilization also fails to interact with such liposomes (Fig. 2c). DSACHe, purified after cholate solubilization, binds similarly to liposomes composed of PS (Fig. 2e) and PC (results not shown), but AChE directly solubilized with PIPLC is unable to bind to PS (Fig. 2f) and PC

Table 2. *Effect of phospholipases and proteinases on the sedimentation coefficient of DSACHe*
AChE (5 μ l, 170 μ g/ml), purified after solubilization in cholate, was incubated with the proteinases or phospholipases listed below, before dilution into 1% Brij 96 (200 μ l). Incubation was carried out in buffer I, unless otherwise stated, under the specified conditions listed below. After dilution, the samples were retained at 22°C for 1 h, and then layered on to 5–20% sucrose gradients prepared in buffer I containing 0.1% Brij 96. The superscripts correspond to the conditions of incubation with the appropriate phospholipase or proteinase: ¹25 μ g/ml, 30 min, 37°C; ²100 μ g/ml, 1 h, 37°C, 10 mM-Ca²⁺; ³200 μ g/ml, 2 h, 37°C, 10 mM-Ca²⁺; ⁴100 μ g/ml, 1 h, 37°C, 50 mM-sodium acetate (pH 6.0)/50 mM-CaCl₂/30 mM-NaCl; ⁵200 μ g/ml, 2 h, 37°C, 50 mM-sodium acetate (pH 6.0)/50 mM-CaCl₂/30 mM-NaCl; ⁶50 or 100 μ g/ml, 30 min, 22°C; ⁷50 μ g/ml, 30 min, 22°C; ⁸100 μ g/ml, 30 min, 22°C; ⁹200 μ g/ml, 30 min, 22°C; ¹⁰500 μ g/ml, 30 min, 22°C.

Treatment	Apparent sedimentation coefficient, s (S)
Control	5.3 $\pm$ 0.2 (6)
PIPLC	7.4 $\pm$ 0.2 (6) ¹
Phospholipase A ₂ (<i>Naja naja</i>)	5.4 ² , 5.1 ³
Phospholipase A ₂ (bee venom)	5.1 ² , 5.2 ³
Phospholipase C (<i>C. welchii</i>)	5.5 ² , 5.3 ³
Phospholipase D (cabbage)	5.0 ⁴ , 5.2 ⁵
Proteinase K	7.0 $\pm$ 0.3 (6) ⁶
Pronase E	5.1/6.7 ⁷ , 5.2/6.9 ⁸ , 5.1/7.1 ^{9*}
Chymotrypsin	5.1 ⁹ , 5.4 ⁹
Trypsin	5.3 ⁷ , 5.0 ⁹
Thermolysin	5.6 ⁷ , 5.3 ⁸ , 5.6 ⁹
<i>S. aureus</i> type VIII proteinase	5.0 ⁷ , 5.3 ⁹
Papain	5.3 ⁹ , 5.1 ¹⁰

*Even at the highest Pronase concentration, approximately one-third of the enzyme activity was found in the lighter (5.1S) peak.

Fig. 1. Solubilization of AChE activity in *Torpedo californica* homogenates by incubation with various proteinases

A particulate fraction of *Torpedo californica* electric organ was prepared as described in the Experimental section, except that two successive washes in the high-salt buffer (2M-MgCl₂/100mM-NaCl/10mM-Tris, pH 8.0) were employed so as to ensure complete removal of high-salt-soluble AChE. The homogenates were incubated in buffer I at 22°C for 30 min with the designated concentrations of the various proteinases. Portions were then centrifuged in a Beckman Airfuge (100000g, 10 min) and AChE activity was measured in the supernatant. In all cases, assay of the whole homogenates revealed no significant loss of total enzymic activity. ----, Control supernatant; ●, Proteinase K; ○, Pronase E; ■, trypsin; □, thermolysin.

liposomes (results not shown). PIPLC is able to solubilize a substantial fraction of the AChE activity from liposomes containing PC, after DSACHe had been incorporated into these liposomes (compare Fig. 2g with 2h), suggesting that, in an artificial lipid bilayer, the PIPLC-sensitive site on the AChE molecule remains largely accessible to PIPLC.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed on unpurified AChE solubilized by PIPLC (Fig. 3a) and on AChE that had been affinity-purified after solubilization with Proteinase K (Fig. 3b). In both cases the active sites were specifically labelled with [³H]DFP. Both preparations reveal a single labelled component of M_r ~135000 before reduction and ~64000 after reduction, values similar to those previously reported for cholate-solubilized DSACHe (Viratelle & Bernhard, 1980). All three forms thus seem to be disulphide-linked catalytic-subunit dimers.

Table 3. M_r of AChE after solubilization by various methods

The M_r of [³H]DFP-labelled AChE was determined as described in the Experimental section by comparison with the [¹⁴C]methylated protein markers. The results are shown as means ± S.E.M., where appropriate.

AChE	$10^{-3} \times$ Average M_r
High-salt-soluble	65.6 ± 0.4 (3)
Low-salt-soluble	63.8 ± 0.9 (6)
PIPLC-solubilized	63.8 ± 0.3 (5)
Cholate-purified	63.25 (2)
Brij 96-solubilized	63.5 (1)
Triton-solubilized	63.0 (2)
Proteinase K-purified	63.2 ± 0.6 (3)

In order to detect small differences in the M_r of the catalytic subunit of different preparations of AChE, samples labelled with [³H]DFP were co-electrophoresed with a mixture of [¹⁴C]methylated proteins. By this technique, samples of DSACHe migrate significantly faster than bovine serum albumin (M_r 66000; see Dayhoff, 1976) and have apparent M_r values of 63000–64000 whether solubilized in cholate, Brij 96 or Triton X-100 (Table 3). AChE solubilized by either PIPLC or Proteinase K does not have an M_r significantly lower than that of DSACHe. Low-salt-soluble AChE has a similar M_r , but high-salt-soluble AChE migrates significantly more slowly, almost together with the bovine serum albumin marker (Table 3).

Discussion

The results presented above provide strong evidence for involvement of PI (or a derivative thereof) in attachment of the hydrophobic dimer of AChE to the plasma membrane in *Torpedo* electric organ (see the model, Fig. 4a). Furthermore, one or more PI molecules must be co-solubilized with DSACHe in cholate (Fig. 4b) and remain tightly attached to the enzyme throughout purification, since we have clearly shown that PIPLC modifies the physicochemical behaviour of the purified enzyme. Thus, after digestion of cholate-purified DSACHe with PIPLC, the sedimentation coefficient of DSACHe is no longer detergent-sensitive.

The effects of PIPLC and Proteinase K on detergent-sensitivity parallel their ability to solubilize the hydrophobic dimer of AChE from *Torpedo* homogenates (Futerman *et al.*, 1983). Thus the site of detergent binding corresponds to, or overlaps with, the site of interaction of the enzyme molecule

Fig. 2. Interaction of DSACHe with liposomes of defined composition

Flotation gradients were prepared and centrifuged as described in the Experimental section. (a) Cholate-purified DSACHe in the absence of liposomes. (b) Cholate-purified DSACHe with PC/PI liposomes. (c) AChE, purified after solubilization with Proteinase K, with PC/PI liposomes. (d) Cholate-purified DSACHe after PIPLC treatment (25 µg/ml, 37°C, 30 min) with PC/PI liposomes. (e) Cholate-purified DSACHe with PS liposomes. (f) AChE, solubilized by PIPLC, with PS liposomes. (g) Cholate-purified DSACHe was mixed with PC liposomes and dialysed overnight at 4°C against a large volume of buffer I before application to the gradient. (h) As for (g), except that dialysis was followed by incubation at 37°C for 30 min with PIPLC (17 µg/ml). In all cases, liposomes floated to the top of the gradient (to the left in the Figure) as measured by monitoring turbidity at 300 nm.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of *Torpedo* AChE solubilized with PIPLC and Proteinase K

Samples of AChE solubilized by PIPLC or purified after solubilization by Proteinase K were labelled with $[^3\text{H}]\text{DFP}$; the samples were then electrophoresed with or without the addition of β -mercaptoethanol (see the Experimental section). Gels were sliced and radioactivity counted as described in the Experimental section. Continuous lines represent non-reduced AChE samples and the broken lines samples reduced with β -mercaptoethanol. (a) AChE solubilized by PIPLC. (b) AChE purified after solubilization with Proteinase K. The arrows denote markers: *E. coli* β -galactosidase (β -GAL; M_r 135 000), bovine serum albumin (BSA; M_r 66 000) and ovalbumin (OVALB; M_r 43 000).

with the plasma membrane, which presumably involves the fatty acyl chains of the tightly bound PI (cf. Figs. 4a and 4b).

Our data on the binding of DSACHe to liposomes are in good agreement with the data obtained by the two above approaches, since both Proteinase K and PIPLC abolish binding. It is of particular interest that DSACHe treated with PIPLC does not bind even to liposomes containing PI. The effect of PIPLC suggests that it either

eliminates a site on the enzyme responsible for binding of DSACHe to the plasma membrane or cleaves a phosphate-glycerol bond in such a manner as to leave a fragment of PI (or a derivative thereof) adhering to the enzyme, which blocks reconstitution (see Fig. 4d). Alkaline phosphatase solubilized from pig kidney microsomes by PIPLC is also unable to bind to liposomes, even if they contain PI, whereas alkaline phosphatase solubilized by butanol does bind (Low & Zilversmit, 1980). The failure of PIPLC-treated AChE and PIPLC-treated alkaline phosphatase to bind to liposomes thus favours the latter possibility that a fragment of PI remains tightly attached to the enzyme after PIPLC treatment. The similar loss of detergent-sensitivity and ability of AChE to bind to liposomes after treatment with Proteinase K might then be ascribed to proteolytic detachment of a peptide to which the PI residue(s) would be strongly attached (Fig. 4c). However, the gel-electrophoresis data (Table 3) revealed no significant difference in apparent M_r between intact DSACHe and the enzyme modified with either PIPLC or Proteinase K. The catalytic polypeptide derived from high-salt-soluble AChE migrated almost together with the bovine serum albumin marker, and was clearly ~ 2 kDa heavier than that of DSACHe. This confirms the observation by Witzemann & Boustead (1983), who similarly compared the corresponding forms of AChE from *Torpedo marmorata*. Furthermore, it shows that any similar change in M_r induced by Proteinase K would have been readily detectable, and sets an upper limit of ~ 1.5 kDa (i.e. 10–15 amino acids) for the putative peptide removed by its action. The fact that of six homogeneous proteinases tested (the seventh, Pronase E, is a mixture) only one, namely Proteinase K, modifies the detergent-sensitivity of DSACHe, also suggests that a relatively small polypeptide sequence is accessible to proteolytic attack.

It is currently believed that integral membrane proteins are usually positioned in the plasma membrane by one or more transmembrane hydrophobic polypeptide sequences that are in direct contact with the hydrocarbon chains of the membrane lipids (Capaldi, 1982). Many such proteins are amphipathic molecules, and proteolytic digestion permits separation of the functional part, as a water-soluble entity, from the hydrophobic anchor. This anchor can be derived from C-terminal, N-terminal or intermediate sequences of the polypeptide chain (Macnair & Kenny, 1979; Capaldi, 1982; Kensil *et al.*, 1983). Such sequences must contain at least 20–25 amino acids in order to span the bilayer, and thus should have a minimal M_r of ~ 2500 ; indeed, typical reports describe such hydrophobic fragments ranging in size from 3 kDa

Fig. 4. Schematic representation of modes of solubilization and enzymic transformation of the hydrophobic dimer of AChE. The model assumes that both subunits of the AChE dimer are associated with one or more molecules of PI (or a PI derivative), which serve to anchor the enzyme in the membrane (A) and are responsible for its interaction with detergent on detergent solubilization (B). Proteinase K can directly solubilize membrane-bound AChE (A) by digestion of a small peptide to produce a form that is unable to interact with detergents (C). PIPLC can similarly solubilize membrane-bound AChE directly by digestion of one or more PI molecules to yield a form that is also unable to interact with detergents (D). Both Proteinase K and PIPLC are able to act on detergent-soluble AChE (B) to yield the same detergent-insensitive forms (C and D) as produced by direct solubilization with these enzymes.

upwards (Macnair & Kenny, 1979; Kensil *et al.*, 1983).

In recent years, however, considerable evidence has accumulated for another mode of attachment of integral membrane proteins, involving covalently bound fatty acids (Schlesinger, 1981; Omary & Trowbridge, 1981; Sefton *et al.*, 1982; Henning & Lange-Mutschler, 1983; Ferguson & Cross, 1984). A particularly well studied protein in this respect is the amphiphilic membrane-bound penicillinase from *Bacillus licheniformis*. This enzyme is anchored to the plasma membrane via its *N*-terminal region (Izui *et al.*, 1980), and a water-soluble exoform can be produced by cleavage of the *N*-terminal octapeptide (Chang *et al.*, 1982). According to its amino acid composition, this short peptide is hydrophilic, and cannot, therefore, be responsible for the amphipathic character of the membrane-bound form or for its membrane anchorage. Evidence has been produced (Nielsen *et al.*, 1981; Lai *et al.*, 1981) that this is achieved by covalent modification of both the thiol and NH_2 groups of the *N*-terminal cysteine residue by a diacylglycerol and a fatty acid respectively. Our data suggest that tightly bound PI (or a derivative thereof) may similarly suffice to anchor *Torpedo*

AChE to the plasma membrane, since PIPLC is able to remove the enzyme swiftly and quantitatively, and the studies with proteinases suggest that only a small polypeptide is involved. It is worth noting that both for intestinal alkaline phosphatase (Colbeau & Maroux, 1978) and human erythrocyte AChE (Dutta-Choudhury & Rosenberry, 1984), relatively small anchoring peptides, 3 and 2 kDa respectively, have been reported; furthermore, both alkaline phosphatase (Low & Finean, 1977b) and erythrocyte AChE (Low & Finean, 1977a; Futerman *et al.*, 1985), are solubilized by PIPLC, although only partially in the case of human erythrocyte AChE.

If the analogy with the bacterial penicillinase holds fully, a stoichiometric amount of PI might be covalently attached to the catalytic subunit of AChE, as shown in the model in Fig. 4.

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