Structure of the glycosyl-phosphatidylinositol membrane anchor of acetylcholinesterase from the electric organ of the electric-fish, *Torpedo californica*

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The structure of the glycan moiety of the glycosyl-phosphatidylinositol (GPI) membrane anchor from *Torpedo californica* (electric fish) electric-organ acetylcholinesterase was solved using n.m.r., methylation analysis and chemical and enzymic micro-sequencing. Two structures were found to

be present: Glc α 1-2Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6myo-inositol and Glc α 1-2Man α 1-2Man α 1-6(GalNAc β 1-4)Man α 1-4GlcN α 1-6myo-inositol. The presence of glucose in this GPI anchor structure is a novel feature. The anchor was also shown to contain 2.3 residues of ethanolamine per molecule.

INTRODUCTION

Glycosyl-phosphatidylinositol (GPI) membrane anchors are ubiquitous in the eukaryotic kingdom (Ferguson and Williams, 1988; Low, 1989; Cross, 1990; Ferguson, 1991, 1992a). Their primary function is to afford the stable association of certain glycoproteins with the exoplasmic face of the plasma membrane, or the topologically equivalent luminal face of secretory granules. However, their involvement in other functions, such as potocytosis (Anderson et al., 1992), transmembrane signalling (Robinson, 1991) and intracellular targeting (Lisanti and Rodriguez-Boulant, 1991) have also been described. The biosynthesis of GPI anchors has also been extensively reviewed (Caras, 1991; Menon, 1991; Udenfriend et al., 1991; Tartakoff and Singh, 1992; Englund, 1993). A book on the subject of GPI anchors was recently published (Cardoso de Almeida, 1992).

To date complete or partial structures for GPI anchors have been determined from protozoan (Ferguson et al., 1988; Schneider et al., 1990; Güther et al., 1992), yeast (Fankhauser et al., 1993) and mammalian (Homans et al., 1988; Deeg et al., 1992; Stahl et al., 1992) sources. The consensus structure of GPI anchors is shown in Figure 1, which indicates the known conserved and variable features of this class of molecules. In this paper we describe the structure of the GPI anchor isolated from the dimeric (G_2) form of acetylcholinesterase (AChE) purified from the electric organ of the electric fish, *Torpedo californica*. This form of AChE, which is associated with the presynaptic membrane (Eichler et al., 1992), was one of the first proteins for which chemical evidence was provided for the presence of a PI-containing membrane anchor (Futerman et al., 1985; Low et al., 1986).

EXPERIMENTAL

Materials

AG3X4, AG 50X12 and Bio-Gel P4 resins were from Bio-Rad; quaternary aminoethyl (QAE)-Sephadex and Superose 12HR were from Pharmacia. Proteinase K (protease type XI), phenyl-

methanesulphonyl fluoride, jack-bean (*Canavalia ensiformis*) β -hexosaminidase and yeast α -glucosidase were from Sigma. Jack-bean α -mannosidase was from Boehringer, and *scyllo*-inositol was from Calbiochem. $^2\text{H}_2\text{O}$ (Gold grade) was from Aldrich, 6 M HCl was from Pierce Chemical Co., and the NaB^3H_4 (sp. radioactivity 10–15 Ci/mmol) was from du Pont–New England Nuclear. Centricon-30 microconcentrators were from Amicon Corporation (Danvers, MA, U.S.A.), SEP-PAK C_{18} cartridges were from Waters (Milford, MA, U.S.A.), and nitrocellulose filters were from Falcon. *Bacillus thuringiensis* PI-specific phospholipase C (PI-PLC) was generously given by Dr. Martin Low, Columbia University, New York, NY, U.S.A. Glucosidases I and II from liver were generously given by Dr. Terry Butters (Oxford Glycobiology Unit, Oxford, U.K.).

Purification of the PI-PLC-treated AChE GPI anchor (fraction A)

AChE was purified from frozen *Torpedo californica* electric-organ tissue (Marinus, Long Beach, CA, U.S.A.), after solubilization of the enzyme with PI-PLC, by an affinity-chromatography procedure (Futerman et al., 1985; Sussman et al., 1988). Typically 8–10 mg of protein was obtained from 500–600 g of frozen tissue. The enzyme was concentrated to 10 mg/ml in a Centricon-30 microconcentrator, then treated with proteinase K [15% (w/w) of AChE in 50 mM ammonium acetate, pH 6.8]. After 1 h at room temperature, digestion was stopped by adding phenylmethanesulphonyl fluoride to a final concentration of 2 mM. The digest was freeze-dried, reconstituted in 0.28 ml of 0.1 M ammonium acetate and passed through a 0.22 μm -pore-size nitrocellulose filter prior to gel filtration on a Superose 12HR column (1 cm $\times$ 30 cm); elution was with 0.1 M ammonium acetate, pH 6.8, at 0.3 ml/min. The eluate was monitored for absorbance at 225 nm, and 0.3 ml fractions were collected and assayed for total phosphorus content. The phosphorus-containing fractions (which were eluted at about 55 min) were pooled, freeze-dried, redissolved in 2 ml of 0.1% trifluoroacetic acid (TFA) and passed through a SEP-PAK C_{18} cartridge. The cartridge was washed with 2 ml of 0.1% TFA, then with

Abbreviations used: AChE, acetylcholinesterase; GPI, glycosyl-phosphatidylinositol; TFA, trifluoroacetic acid; PMAA, partially methylated alditol acetate; Du, Dionex units; Gu, glucose units; AHM, 2,5-anhydromannitol; PI-PLC, PI-specific phospholipase C; QAE, quaternary aminoethyl; COSY, correlation spectroscopy; ROESY, rotating-frame nuclear Overhauser spectroscopy; AHM, 2,5-anhydromannitol.

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Figure 1 Consensus structure of GPI protein anchors

The conserved backbone structure can be variously substituted with carbohydrate and/or ethanolamine phosphate (EINP) as indicated by the R¹–R⁴ groups. In the case of *Trypanosoma (T.) brucei* PARP (*) the position(s) of carbohydrate substitution are unknown. Other abbreviations: VSG, variant surface glycoprotein; PARP, procyclic acidic repetitive protein; PSP, promastigote surface protease; AChE, acetylcholinesterase; Prp, prion protein; L., Leishmania; SA, sialic acid.

Anchor	R ¹	R ²	R ³	R ⁴	Lipid	Palmitate	Reference
<i>T. brucei</i> VSG	–	α-Gal ₆₋₇	–	–	Diacylglycerol	–	Ferguson et al. (1988)
<i>T. brucei</i> PARP	[	SA ₅ Gal ₅ GlcNAC ₉	–	]*	LysoAcylglycerol	+	Field et al. (1991)
<i>L. major</i> PSP	–	–	–	–	Alkylacylglycerol	–	Ferguson et al. (1993)
<i>T. cruzi</i> 1G7	–	–	–	α-Man	Alkylacylglycerol	?	Schneider et al. (1990)
Yeast proteins	–	–	–	α-Man ₁₋₂	Ceramide	–	Güther et al. (1992)
Rat brain Thy-1	EINP	β-GalNAc	–	± αMan	Alkylacylglycerol(?)	–	Fankhauser et al. (1993)
Human AChE	EINP	–	± EINP	–	Alkylacylglycerol	+	Homans et al. (1988)
Hamster Prp	EINP	SA-Gal-GalNAc	–	± Man	Diacylglycerol (?)	–	Roberts et al. (1988); Deeg et al. (1992)

increasing concentrations of acetonitrile in 0.1% TFA. The phosphorus-containing fractions were found in the initial aqueous eluate, whereas material absorbing at 225 and 280 nm was recovered in the acetonitrile-containing eluates. The phosphorus-containing fractions were pooled, freeze-dried, redissolved in 0.1 ml of aq. 0.1% TFA and subjected to h.p.l.c. using an RP-18 reverse-phase column (Merck LiChrosphere 100RP; end-capped; 10 µm particle size; 25 cm × 0.4 cm). Column elution was isocratic at 0.3 ml/min in 0.1% TFA for 20 min, followed by a linear gradient up to 100% acetonitrile over 10 min. Phosphorus-containing fractions, which were unretarded by the column, were pooled and constituted fraction A (Figure 2).

N.m.r. analysis of fraction A

Two-dimensional homonuclear ¹H correlation spectroscopy (COSY) and ¹H rotating-frame nuclear Overhauser spectroscopy (ROESY) spectra were recorded at 500 MHz and a probe temperature of 300 K. In COSY spectra, a total of 1024 *t*₁ increments were recorded, with 64 scans per *t*₁ increment and 4096 complex points in *t*₂, whereas 512 *t*₁ increments and 2048 complex points were acquired in *t*₂ for ROESY spectra. Prior to two-dimensional Fourier transformation, the data were multiplied in each dimension by unshifted sine-bell functions in COSY spectra, or by cosine-bell functions in ROESY spectra, followed by zero-filling once in *t*₁.

Compositional analyses

Total phosphorus was measured by the method of Bartlett (1959). Amino acids, ethanolamine and hexosamines were measured following hydrolysis (6 M HCl, *in vacuo*, 6 h, 115 °C) on a Biotronic LC 5000 amino acids analyser, using an extended program which facilitated separation and quantification of the amino sugars and of ethanolamine. Inositol, monosaccharides and hexose 6-phosphates were measured by g.c.–m.s. as described previously (Ferguson, 1992b). N-terminal amino acids were determined using an Applied Biosystems 120A phenylthiohydantoin analyser.

Dephosphorylation and N-acetylation of fraction A

Fraction A (20 nmol of *myo*-inositol) was dephosphorylated in 50 µl of aq. 50% HF at 0 °C for 60 h. After neutralization with saturated LiOH and centrifugation, the supernatant and pellet washings were combined (Ferguson, 1992b). Half of this material was adjusted to 1 M NaHCO₃ with solid NaHCO₃ and N-acetylated by the addition of acetic anhydride at 0 °C (three additions of 10 µl acetic anhydride at 10 min intervals). The N-acetylated glycan core was desalted by passage through a column of 0.8 ml of AG50X12 (H⁺ form) resin over 2.5 ml of AG3X4 (OH[–] form) resin over 0.2 ml of QAE-Sephadex A-25. This material, fraction B (Figure 2), was dried and used for further analysis.

Figure 2 Reaction scheme of *Torpedo* AChE GPI anchor analysis

The treatments used included aqueous dephosphorylation (Aq. HF), deamination and NaB₃H₄ reduction (HONO/NaB₃H₄), N-acetylation (N-Ac), TFA hydrolysis (TFA) and jack-bean β -hexosaminidase digestion (JBBH). Key: EtN, ethanolamine; $\odot$, phosphate; $\blacktriangleleft$, Glc; $\circ$, Man; $\square$, GalNAc; $\boxtimes$, GlcNH₂; $\blacksquare$, GlcNAc; $\diamond$, inositol; $\triangleleft$, AHM.

Methylation analysis

Methylation was performed on 50% of Fraction B (Figure 2) using a modification of the method of Ciucanu and Kerek (1984). Analysis of the resulting partially methylated alditol acetates (PMAAs) was by g.c.-m.s. as described by Ferguson (1992b). G.c.-m.s. analyses were performed using both SE-54 and SP2380 bonded-phase columns, for the detection of hexosamine PMAAs and for resolution of mannose and glucose PMAAs respectively.

Nitrous acid deamination and NaB₃H₄ reduction

The remaining 50% of the combined supernatants from aq. HF dephosphorylation of fraction A (see above) were freeze-dried and redissolved in 50 μ l of water. An insoluble LiF residue was removed by centrifugation. The supernatant was dried again and dissolved in 30 μ l of 100 mM sodium acetate, pH 4.0, to which was added 30 μ l of freshly prepared 0.5 M sodium nitrite. After 2.5 h at room temperature, 10 μ l of 0.8 M boric acid was added

and the pH adjusted to 11 with 1 M NaOH (Ferguson, 1992b). Immediately after adjusting the pH, 5 μ l of 36 mM NaB₃H₄ (10–15 Ci/mmol), dissolved in 0.1 M NaOH, was added. After incubation for 90 min, 10 μ l of 1 M NaB₃H₄ was added and incubation continued for another 3 h. The reduction mixture was acidified with 1 M acetic acid, passed through a column of 0.2 ml of AG50X12 (H⁺ form), dried and evaporated twice with 0.25 ml of 5% acetic acid in methanol, then twice with 0.25 ml of methanol to remove boric acid. The labelled neutral glycans generated by this procedure were purified from radiochemical contaminants by paper chromatography and high-voltage electrophoresis as described by Ferguson (1992b). The resulting material, fraction C (Figure 2), was analysed by h.p.l.c.

H.p.l.c.

H.p.l.c. was performed with a Dionex Bio-LC carbohydrate analyser equipped with a CarboPac PA-1 column, pulsed amperometric detector, anion micro-membrane suppressor and a Raytest Ramona radioactivity flow monitor. Labelled neutral glycan samples were co-injected with 150 μ g of β -glucose oligomers (dextran partial hydrolysate) and eluted with a linear gradient from 12.5 to 50 mM sodium acetate in 150 mM NaOH over 50 min at 0.6 ml/min (program 1). For the determination of monosaccharides, program 2, an isocratic gradient of 150 mM NaOH, was used. The column was washed with 150 mM NaOH and 250 mM sodium acetate between analyses. The elution positions of labelled neutral glycans are expressed in 'Dionex units' (Du) by linear interpolation of their retention times between those of the adjacent β -glucose oligomer internal standards (Ferguson 1992b). Gel filtration was performed using a column (1.5 cm $\times$ 1 m) of Bio-Gel P4 (–400 mesh) held at 55 $^{\circ}$ C and eluted with water at 0.2 ml/min. Samples were co-injected with 750 μ g of β -glucose oligomers and the eluate was monitored using an Erma refractive-index monitor and a Ramona radioactivity monitor. The size of labelled neutral glycans is expressed in 'glucose units' (Gu) by linear interpolation of their retention times between those of the adjacent β -glucose oligomer internal standards (Ferguson, 1992b).

Exoglycosidase treatments

Jack-bean β -hexosaminidase digestions were performed using 30 μ l of enzyme (4 units/ml) in 0.1 M citrate/phosphate buffer (pH 4.2)/10 mM D-mannonic γ -lactone (mannosidase inhibitor), for 18 h at 37 $^{\circ}$ C. Jack-bean α -mannosidase digestions were performed using 30 μ l of the enzyme (25 units/ml) in 0.1 M sodium acetate, pH 5.0, for 18 h at 37 $^{\circ}$ C. Yeast α -glucosidase digestion was performed using 30 μ l of enzyme (100 units/ml) in 0.1 M sodium phosphate buffer, pH 6.0, for 18 h at 37 $^{\circ}$ C. The above digestions were terminated by heating (100 $^{\circ}$ C, 5 min), and the digests were desalted by passage through 0.2 ml of AG50 (H⁺ form) resin over 0.4 ml of AG3 (OH[–] form) resin over 0.1 ml of QAE-Sephadex A25 (OH[–] form). Liver α -glucosidase I digestions were performed using 20 μ l of enzyme (> 3 μ units/ml) in 0.1 M sodium phosphate buffer (pH 7)/0.8% Lubrol/1 mg/ml BSA/0.1% NaN₃ for 18 h at 37 $^{\circ}$ C. Digests were desalted as described above and extracted with an equal volume of butan-1-ol to remove the detergent. Liver α -glucosidase II digestions were performed using 20 μ l of enzyme (> 12 μ units/ml) in 0.1 M sodium phosphate buffer (pH 7.0)/10% glycerol/0.02% NaN₃ for 18 h at 37 $^{\circ}$ C. After desalting as described above, the sample was dried and passed through a Bio-Gel P4 column (20 cm $\times$ 1.5 cm) to remove the glycerol.

Acetolysis

Compounds for acetolysis were dried in glass, peracetylated in acetic anhydride/pyridine (1:1, v/v) for 30 min at 100 °C, and dried under vacuum. Acetolysis was achieved using 30 ml of acetic anhydride/acetic acid/conc. H₂SO₄ (10:10:1, by vol.) for 6 h at 37 °C. The reaction was stopped by the addition of 10 µl of pyridine and 500 µl of water. The acetylated products were extracted with 250 µl of chloroform, washed three times with 0.5 ml of water, then dried. De-O-acetylation was performed at 37 °C for 60 h in 0.2 ml of methanol and aq. 35% NH₃ (1:1, v/v). The acetolysed neutral glycans were dried and redissolved in water for subsequent chromatographic analysis.

RESULTS

Preparation of the PI-PLC-treated *Torpedo* AChE GPI anchor

AChE was solubilized by PI-PLC treatment of electric-organ tissue and purified by affinity chromatography (Futerman et al., 1985; Sussman et al., 1988). After digestion with proteinase K the soluble C-terminal glycopeptide of *Torpedo* AChE (fraction A, Figure 2) was purified by gel filtration, passage through an SEP-PAC C₁₈ cartridge and reverse-phase h.p.l.c. The compositional analyses of this material (Table 1) showed the presence of *myo*-inositol, ethanolamine and two amino sugars, glucosamine and galactosamine, all typical components of GPI anchors. In addition, a single serine residue was always observed. Serine was the only amino acid residue identified in significant amounts in the first cycle of sequence analysis, recovery being consistent with the presence of a single serine residue; it was also the only amino acid detected in significant, albeit much smaller, amounts in the second cycle. The values for ethanolamine and phosphorus are consistent with the presence of over two ethanolamine phosphate moieties per molecule of anchor. One ethanolamine phosphate moiety is always found attached to the 6-position of the third α -Man residue, in the form of a bridge between the C-terminal amino acid and the anchor (Ferguson, 1992a), an additional ethanolamine phosphate can be found attached to the first α -Man residue in several cases (see Figure 1) and a third ethanolamine phosphate, attached to the second α -Man residue, has been reported present in the anchor of human AChE (Deeg et al., 1992). The low yield of Man in the compositional analysis is consistent with a relatively high degree of ethanolamine phosphate substitution of the Man residues of the AChE anchor, since this renders such residues cryptic, and therefore refractory, to the conventional monosaccharide analysis (Ferguson, 1992b).

N.m.r. spectroscopy of fraction A

The conventional ¹H one-dimensional n.m.r. spectrum (results not shown) showed five anomeric proton signals. However, the ¹H-¹H COSY spectrum (not shown) demonstrated that there were two anomeric signals which overlapped at about 5.1 p.p.m. The COSY spectrum cross-peak multiplicities and chemical shifts of fraction A were remarkably similar to those of the corresponding fraction of the rat brain Thy-1 GPI anchor, which has the structure Man α 1-2[Cys-EtN-PO₄-6]Man α 1-2Man α 1-6[EtN-PO₄-2][GalNAc β 1-4]Man α 1-4GlcNH₂ α 1-6*myo*-inositol-1-PO₄ (where EtN is ethanolamine) (Homans et al., 1988). However, the data indicated that the non-reducing terminal α Man residue in the Thy-1 anchor was replaced by a different residue in the *Torpedo* AChE anchor structure. One of the two overlapping anomeric proton signals at about 5.1 p.p.m. gave

rise to a connectivity network in the COSY spectrum through which the ring-proton resonances were traced as far as C-5. The ³J_{H1,H2} values and characteristic shifts of these resonances suggested that they belonged to an unsubstituted α -Glc residue. In order to determine the sequence of residues in this structure, a ROESY spectrum was recorded (Figure 3). These ROESY data gave essentially the same connectivity information as those derived previously from the NOESY spectrum of the rat brain

Table 1 Compositional analysis of fraction A

Component	Analytical method	Molar ratio
Serine	A.a.a.†	1.0
Ethanolamine	A.a.a.	2.3
Glucosamine	A.a.a.	0.6
Galactosamine	A.a.a.	0.6
Glucose	G.c.-m.s.	0.9
Mannose	G.c.-m.s.	1.0
Mannose-6-phosphate	G.c.-m.s.	0.5
<i>myo</i> -Inositol	G.c.-m.s.	1.2*
Phosphorus	Colorimetric	3.3

† Abbreviation used: A.a.a., amino acid analysis.

* Value based on response factor derived using Man-6-PO₄ as a standard, the yield of the Man-6-PO₄ derivative from ethanolamine-PO₄-Man in a GPI anchor appears to be about 50% (Ferguson et al., 1988).

Figure 3 Reporter region of ¹H-¹H ROESY spectrum of fraction A

The abbreviations labelling each cross-peak correspond to the residue descriptors shown at the top of the Figure, followed by the proton assignment which is labelled according to its parent carbon. Inter-residue connectivities are shown in **bold** type, intra-residue connectivities in Roman type.

Table 2 Methylation linkage analysis of fraction B

PMAA	Origin	Peak area*
1,5-Diacetyl-2,3,4,6-tetramethylglucitol	Terminal Glc	1.0
1,2,5-Triacetyl-3,4,6-trimethylmannitol	2-O-subst.†-Man	1.6
1,5,6-Triacetyl-2,3,4-trimethylmannitol	6-O-subst.-Man	0.4
1,4,5,6-Tetra-acetyl-2,3-dimethylmannitol	4,6-di-O-subst.-Man	0.8
1,5-Diacetyl-3,4,6-Trimethyl-2-N-methylacetamidogalactosaminitol	Terminal GalNAc	1.0
1,4,5-Triacetyl-3,6-dimethyl-2-N-methylacetamidoglucosaminitol	4-O-subst.-GlcNAc	0.9
6-Acetyl-1,2,3,4,5-pentamethyl- <i>myo</i> -inositol	6-O-subst.-inositol	0.7

* Total ion-current peak area.

† Abbreviation: subst., substituted.

Thy-1 GPI anchor (Homans *et al.*, 1988). However, instead of an inter-residue NOESY connectivity corresponding to the terminal Man α 1-2Man sequence, we observed a ROESY connectivity corresponding to a terminal Glc α 1-2Man sequence in the *Torpedo* anchor structure. Thus the ROESY connectivity network is consistent with the sequence Glc α 1-2Man α 1-6Man α 1-4GlcNH $_2$ α 1-6*myo*-inositol. As in the Thy-1 studies, the linkage position of the GalNAc residue could not be determined from the n.m.r. data. However, the presence of a heteronuclear ^1H - ^{31}P splitting on the C-2 proton of the mannose residue in the Man α 1-4GlcNH $_2$ sequence could be confirmed by broad-band phosphorus decoupling (results not shown). This suggests that the C-2 position of this residue may be substituted with ethanolamine phosphate, as in the rat brain Thy-1 GPI anchor.

Methylation linkage composition of fraction B

The methylation analysis of fraction B (Table 2) was consistent with the n.m.r. data on fraction A. Terminal-Glc, 2-O-substituted Man, 6-O-substituted Man, 4-O-substituted GlcNAc and 6-O-substituted *myo*-inositol derivatives were all present, as predicted, but, in addition, terminal-GalNAc and 4,6-di-O-substituted Man were observed. These latter derivatives would be consistent with the substitution of some of the first Man residues with GalNAc at the 4-position, converting the 6-O-substituted Man to 4,6-di-O-substituted Man.

Chromatographic analysis of fraction C

The labelled neutral glycans (fraction C, Figure 2) were separated by Dionex h.p.l.c. using program 1. Aliquots of the three peaks obtained (Figure 4) were hydrolysed (2 M TFA; 100 °C; 3 h) and the hydrolysates analysed by Dionex h.p.l.c. using program 2. For the earliest-to-be-eluted peak the radioactivity was shown not to be co-eluted with authentic 2,5-anhydromannitol (AHM), an obligatory component of deaminated/reduced GPI molecules, and so this peak was not analysed further. In contrast, the other two peaks (eluted at 3.6 Du and 4.2 Du) produced radiolabelled AHM after acid hydrolysis. The fractions containing these peaks were pooled separately, desalted, and analysed by Bio-Gel P4 chromatography, where they were eluted as single peaks of 5.1 Gu and 6.4 Gu respectively. Both peaks were digested with jack-bean β -hexosaminidase and re-analysed by Dionex h.p.l.c. (program 1) and Bio-Gel P4 chromatography. The 3.6 Du/5.1 Gu peak was resistant to digestion, whereas the 4.2 Du/

Figure 4 Dionex h.p.l.c. of the GPI neutral glycans (fraction C)

Radiolabelled neutral glycans were detected using an on-line radioactivity monitor. The values at the top of the Figure (in Du) indicate the elution positions of the co-injected glucose oligomer internal standards.

6.4 Gu peak was eluted at 3.6 Du and 5.1 Gu after digestion. This suggested that the two structures differed by the presence of a single terminal β -HexNAc residue linked to a common 3.6 Du/5.1 Gu core.

Both the 3.6 Du/5.1 Gu and 4.2 Du/6.4 Gu species were resistant to digestion with jack-bean α -mannosidase, indicating that no terminal α -Man residues were available to the enzyme. This was consistent with the n.m.r. and methylation data, which suggested a terminal α -Glc residue instead. However, attempts to digest both species with yeast α -glucosidase and rat liver α -glucosidases I and II were unsuccessful. However, partial acetylation of the 3.6 Du/5.1 Gu core, which selectively cleaves the Man α 1-6Man bond, produced a glycan species which was eluted at 1.1 Du and 2.3 Gu. These chromatographic properties are identical with those of authentic Man α 1-4AHM (Ferguson, 1992b).

Thus the chromatographic data alone define fraction C as:

Taken together with the methylation-analysis and n.m.r. data, they are consistent with the glycan structures shown in Figure 5. Integration of the 3.6 Du and 4.2 Du peaks (Figure 4) indicates that they represent 47 and 53% of the total anchor glycans respectively. This value should be corrected for approx. 15% loss of GalNAc, due to partial de-N-acetylation during aq. HF dephosphorylation followed by deamination (Homans *et al.*, 1988). Using the corrected values, it can be estimated that 62% of the structures contain the β -GalNAc residue.

DISCUSSION

The structure of the *Torpedo* AChE GPI anchor is shown in Figure 5. This is the first description of a GPI anchor structure from a cartilaginous fish. The diacylglycerol structure of the PI moiety is taken from the work of Bütikofer *et al.* (1990), which confirms an earlier report of stearic acid as the dominant fatty acid in the GPI anchor of *Torpedo* AChE (Silman *et al.*, 1989). The overall structure is consistent with the notion of a conserved

The locations of the ethanolamine phosphate substituents ($\text{PO}_4\text{-EtNH}_2$; boxed) are shown by analogy with other GPI examples (Figure 1). The lipid moiety is shown as a diacylglycerol using the data of Bütikofer et al. (1990). Approx. 62% of the structures contain the $\beta\text{-GalNAc}$ residue.

ethanolamine-PO₄-6Man α 1-2Man α 1-6Man α 1-
4GlcN α 1-6*myo*-inositol-1-PO₄-lipid (see Figure 1)

In common with the GPI anchors of all other metazoan organisms so far analysed, and in contrast with all unicellular examples, the *Torpedo* structure contains more than one ethanolamine phosphate group (2.3 mol/mol, according to compositional analysis). One of these was assigned by the n.m.r. data to the 2-position of the α Man residue in the Man α 1-4GlcN sequence. Another presumably serves as the bridge between the GPI anchor and the AChE polypeptide, by analogy with other GPI anchors, and it is possible that a small percentage of the *Torpedo* AChE anchors bear a third ethanolamine phosphate, as observed in human erythrocyte AChE (Deeg et al., 1992) (see Figure 1).

putative GPI attachment site more in keeping with recent analyses of glycolipid-anchoring signals (Caras, 1991; Udenfriend et al., 1991; Kodukula et al., 1993), and would result in a residual C-terminal sequence more amenable to proteolytic cleavage.

A.M., S.W.H. and M.A.J.F. are supported by The Wellcome Trust, and I.S. by the Israel Academy of Sciences and the Minerva Foundation. M.A.J.F. is a Howard Hughes International Research Scholar, I.S. is the Bernstein-Mason Professor of Neurochemistry, and S.W.H. is a Lister Research Fellow. We thank Martin Low for his gift of PI-PLC and Terry Butters for his gift of liver α -glucosidases I and II.

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