Structural Characterization of the Glycoinositol Phospholipid Membrane Anchor of Human Erythrocyte Acetylcholinesterase by Fast Atom Bombardment Mass Spectrometry*

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The glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase (EC 3.1.1.7) is composed of a glycans linked through a glucosamine residue to an inositol phospholipid that is resistant to the action of phosphatidylinositol-specific phospholipase C. Deamination cleavage of the glucosamine with nitrous acid released the inositol phospholipid which was purified by high performance liquid chromatography. Analysis by fast atom bombardment mass spectrometry with negative ion monitoring and by the complementary technique of collision-induced dissociation revealed molecular and daughter ions that indicated a plasmamylinositol with a palmitoyl group on an inositol hydroxyl. The intact membrane anchor was released from reductively methylated human erythrocyte acetylcholinesterase by proteolysis with papain or Pronase, deacylated by base hydrolysis, and purified by high performance liquid chromatography. Positive and negative ion fast atom bombardment mass spectrometry of the major products isolated by high performance liquid chromatography indicated the following structure for the complete glycoinositol phospholipid anchor.

Methylation of free amino groups by reduction with deuterium instead of hydrogen permitted determination of the number of free amino groups in individual fragment ions as further confirmation of structural assignments. The structure of the glycan portion of the human erythrocyte acetylcholinesterase membrane anchor appears to be similar to that described for Trypanosome brucei variant surface glycoprotein MITat 1.4 (variant 117) (Ferguson, M. A. J., Homans, S. W., Dwek, R. A., and Rademacher, T. W. (1988) Science 239, 753–759) except for the absence of a galactose antenna and the presence of a phosphorylethanolamine on the hexose adjacent to glucosamine.

Acetylcholinesterase (EC 3.1.1.7) exists in multiple molecular forms which are widely distributed in many tissues (for a review, see Toutant and Massoulié, 1987). AChE on the surface of human erythrocytes is an amphipathic globular dimeric protein that is anchored in the plasma membrane by a covalently attached glycoinositol phospholipid (Roberts and Rosenberry, 1986; Haas et al., 1986; Roberts et al., 1987; Roberts et al., 1988 (the accompanying paper)). Similar glycolipid anchors have been identified in many proteins, the best characterized of which are trypanosome variant surface glycoproteins (VSGs) (Ferguson and Williams, 1988; Ferguson et al., 1988). Common structural features of glycoinositol phospholipid anchors are an ethanolamine residue in amide linkage to the C terminus of the protein, phosphate groups, a phosphorylethanolamine, and sometimes a galactose antenna.

† The abbreviations used are: AChE, acetylcholinesterase; CID, collision-induced dissociation; E111, human erythrocyte; FAB, fast atom bombardment; HPLC, high performance liquid chromatography.
mannotose-containing glycan, and a nonacetylated glucosamine residue in glycosidic linkage to an inositol phospholipid. In many cases, although only to a slight extent in E° AChE, this phosphoryl can be cleaved by phosphorylidyinositol-specific phospholipase F from *Staphylococcus aureus* to release a soluble hydrophilic derivative of the anchored protein (for a review, see Low, 1987).

Structural studies of glycoinositol phosphoryl anchor proteins have utilized several cleavage procedures to produce anchor fragments for analysis. Papain digestion of E° AChE produced a C-terminal dipeptide His-Gly linked through ethanola mine to the anchor (Roberts and Rosenberry, 1986; Haas et al., 1986). This proteolysis fragment also contained an additional ethanola mine and a glucosamine with unblocked amino groups (Haas et al., 1986) and 2 fatty acid residues (Roberts and Rosenberry, 1985). Treatment of anchored proteins with nitrous acid deaminates the anchor glucosamine and cleaves its glycosidic linkage to inositol (Ferguson et al., 1985a). Deamination of trypanosome VSGs released dimyristoyl phosphorylidyinositol, but deamination of E° AChE generated a novel inositol phosphoryl phospholipid in which an inositol hydroxy group appeared to be palmitoylated (Roberts et al., 1988). This palmitoylation was shown to be responsible for the resistance of the E° AChE anchor to phosphorylidyinositol-specific phospholipase C from *S. aureus* (Roberts et al., 1988).

Fast atom bombardment (Barber et al., 1981; Morris et al., 1982; Reinhold, 1986; Dell, 1987) is a soft ionization process for mass spectrometry that is characterized by the production of abundant molecular weight-related ions, (M + H)+, (M – H)–, and (M + Na)+, of intact molecules that are termed “parent” ions. A small fraction of these parent ions have sufficient energy to undergo fragmentation providing added structural detail to the FAB mass spectrum. Fragmentation can be enhanced by parent ion collision with a neutral gas (helium, for example) in a process referred to as collision-induced dissociation (CID). Mass spectrometers with multiple analyzers can combine the processes of parent ion focusing, CID in an intermediate zone, and subsequent product or “daughter” ion analysis. The recording of daughter ion mass spectra of preselected precursor ions also offers advantages in eliminating matrix contributions and confirming that fragment ions in the FAB spectra indeed arise from a particular parent (Biemann and Martin, 1987).

In this paper FAB-MS was used with negative ion extraction to characterize the structure of the inositol phosphoryl phospholipid product released from E° AChE by nitrous acid deamination. Structural assignments were confirmed by daughter ion analysis for CID. FAB-MS analysis of the glycoinositol phospholipid anchor released by proteolysis provided the molecular weight and additional information about the anchor structure. The sensitivity of this technique made it ideal for the small quantities of sample available.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Radiolabeling—AChE was extracted from outdated human erythrocytes with Triton X-100 and purified by affinity chromatography on acridinium resin (Roberts et al., 1987). Where indicated, the purified enzyme was radiolabeled with [**32P**]ITID (Amersham Corp.) (Roberts and Rosenberry, 1986) or radiomethylated with [**14C**]CHCHO in Triton X-100. In a slight modification of a previous procedure (Haas et al., 1986). Briefly, samples of AChE (100–200 nmol) to which a trace amount of [**14C**]labeled enzyme had been added as a specific marker for the membrane anchor were concentrated 10–25-fold in a Speedvac concentrator (Savant Instruments, Inc.) and reductively methylated by incubation with 10 mM [**14C**]CHCHO (ICN, 1-4 mCi/mmol, by dilution with unlabeled HCHO) and 50 mM NaCNBH₃ (or where noted NaCNBD₃) at 37 °C for 15 min. After reductive methylation the samples were dialyzed extensively and repurified by affinity chromatography on acridinium resin (Haas et al., 1986).

**Papain Proteolysis—**Repurified radiomethylated AChE (280 nmol) was dialyzed extensively and digested with activated papain resin (Dutta-Choudhury and Rosenberry, 1984). The anchor-containing proteolysis product was isolated by passage through acridinium resin and gel exclusion chromatography on Sepharose CL-6B and Sephadex LH-60 (Roberts and Rosenberry, 1986).

**Pronase Proteolysis—**Repurified radiomethylated AChE (100–200 nmol), reduced either with NaCNBH₃ or NaCNBD₃, was dialyzed against water and reduced in volume in a Speedvac concentrator prior to incubation for 10 h at 50 °C in a 3:1 mixture of Pronase (10 mg/ml), 0.1 M Hepes (pH 8.0), 15 mM CaCl₂, and 1% Triton X-100. After the addition of 1.5 mM NaDodSO₄ was collected. The anchor-containing proteolysis product had an apparent molecular mass of about 3 kDa relative to peptide standards (Roberts and Rosenberry, 1986). The peak fractions were pooled, concentrated to 200 μl, mixed with 1 ml of formic acid, and applied to a Sephadex LH-60 column (1.5 × 70 cm) equilibrated in ethanol, 85% formic acid (2 ml) and 1.5 ml fractions were collected. The anchor-containing proteolysis product had an apparent molecular mass of about 15 kDa relative to peptide standards (Roberts and Rosenberry, 1986).

**Alkaline Hydrolysis and HPLC Purification of the Papain and Pronase Proteolysis Products—**Anchor-containing proteolysis products purified on Sephadex LH-60 were dried in a Speedvac concentrator and dissolved in 0.1 N aqueous NaOH. The samples were incubated 16 h at 4 °C followed by acidification with 10–50 μl of acetic acid. The base-hydrolyzed papain proteolysis product was chromatographed by reverse phase HPLC on a 4.6 × 250-mm, wide-pore C₅ column (Supelco, Inc.) with a linear gradient of water/1-propanol (0.1% trifluoroacetic acid) from 20 to 60% 1-propanol over 80 min at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected, and radioactivity was monitored by liquid scintillation counting a 10-μl aliquot from each fraction. The base-hydrolyzed Pronase proteolysis product was analyzed by reverse phase HPLC, using a 4.6 × 150-mm proprietary C₁₅ column (Waters Associates; 300 Å pore, 10 μm particle size). A linear gradient described above for the C₁₅ column was applied, 200-μl fractions were collected, and 5-μl aliquots were counted by liquid scintillation. The C₁₅ column was found to give better recoveries of radioactivity (90% versus 75% for the C₁₀ column) and narrower peaks (<1 ml versus 2 ml for the C₁₀ column), the latter largely due to the reduced column volume.

**Neutral Modification of the Nitric Acid Deamination Product—**[**14C**]ITID-labeled E° AChE was treated with nitrous acid, and products extracted into an organic phase were chromatographed by HPLC (Roberts et al., 1988). Isolated deamination product (2 nmol) was acetylated by adding pyridine/acetic anhydride (1:1) (20 μl) and incubating for 1.5 h at room temperature. The acetylated product (2 nmol) was deacetylated by the addition of ammonium-saturated methanol and incubation for 2 h at 65 °C. Both samples were dried under vacuum and used without further purification.

**Mass Spectrometry—**FAB mass spectra were recorded on a VG Analytical ZAB-SE instrument operating at 8 kV. Dried samples (1-3 nmol) were dissolved in 2–5 μl of either dichloromethane (deamination product and derivatives) or methanol (proteolysis products) and deposited onto the FAB target which was thinly coated with a liquid matrix (1–2 μl, glycerol for positive ion spectra and triethanolamine for negative ion spectra). The sample was then bombarded with a 7-kV neutral xenon beam. The mass was scanned at 30 s per decade, and 5 to 6 scans were acquired and averaged on a VG 11250 data system. In CID experiments the precursor ions were focused into the collision cell which was pressurized with helium until the precursor ion abundance was reduced by 50%. The product ions were analyzed by scanning the electrostatic analyzer linearly from 8200 to 25 V over 30 s, a technique referred to as mass-analyzed ion kinetic energy spectroscopy (MIKES). Approximately five scans were acquired in the multichannel averaging mode and smoothed for presentation.

**RESULTS**

**The Nitric Acid Deamination Product Is a Plasmanylinositol**

Plasmanylinositol is an analog of phosphorylidyinositol in which glycerol has one O-linked alkyl and one O-linked acyl group.
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Group—E°chu AChE labeled on the anchor lipid with a trace amount of [128]TID (Roberts et al., 1987) was treated with nitrous acid and the released inositol phospholipid was purified by HPLC (Fig. 1). A major peak of radioactivity was well separated from unlabeled UV-absorbing peaks that represented residual Triton X-100. The labeled peak contained 2 mol of fatty acid/mol of myo-inositol, consistent with the stoichiometry reported in the accompanying paper (Roberts et al., 1988). Direct analysis of this purified product by negative ion FAB-MS (Fig. 2A) revealed several clusters of molecular weight-related ions (M—H)~ with a predominant group containing two major ions at m/z 1135.7 and 1137.7. The adjacent ions and clusters could be attributed to lipid heterogeneity arising from alkyl and acyl chain unsaturation (Δ ± 2, Δ ± 4) and chain length variations in the number of methylene groups (Δ ± 14, Δ ± 28), a feature characteristic of naturally occurring phospholipids. The observed molecular ion heterogeneity was consistent with the alkyl and acyl chain composition determined for the inositol phospholipid (Roberts et al., 1988). Moreover, fragment ions shown below to contain lipid could be distinguished by their heterogeneity (e.g. m/z 735.5) from more homogeneous fragments free of lipid (e.g. m/z 497.2 and 537.2).

To establish the structure of the inositol phospholipid generated from E°chu AChE by nitrous acid treatment in greater detail, the most intense molecular ion (M—H)~ (m/z 1135.7, Fig. 2A) produced by FAB ionization was focused into a helium-filled collision cell, and the daughter ions were studied in a two-sector mass spectrometer (Fig. 2B). Since fragments in this MIKES analysis can originate only from the focused parent ion (or isobaric components), this method eliminates the possibility of contaminants contributing to the spectrum. Utilization only of energy focusing resulted in a mass window that was several atomic mass units wide, but peak centroid analysis provided nominal mass assignments adequate to resolve the major fragments. Predominant fragments in the FAB mass spectrum (Fig. 2A) correspond to those in the MIKE spectrum (Fig. 2B) and thus clearly derive from the m/z 1135.7 and 1137.7 molecular ions. To ascertain further the daughter ion composition of these fragments, each ion in the FAB spectrum (Fig. 2A) isobaric with a daughter ion in the MIKE spectrum was also analyzed by MIKES. The products of this analysis frequently produced single constituents or conjugate groups, and this lineage relationship assisted in composing the final structure. For example, MIKE analysis of the nitrous acid deamination product ((M — H)~, m/z 1135.7) produces a daughter ion fragment of m/z 736. The difference in mass, 400 Da, corresponds to a palmitoylated inositol group that was eliminated with a hydrogen transfer back to the ruptured bond. When the m/z 735.5 ion generated by FAB was subjected to CID, the MIKE spectrum indicated a daughter ion m/z 405.2 that arose by elimination of the acyl group at the 2-position of glycerol. Further study of the m/z 405.2 ion produced by FAB (Fig. 2B) by CID-MIKES indicated a daughter ion equal in mass to an ionized dihydrogen phosphate group (m/z 97). Thus, the m/z 405.2 ion can be reconstructed as an 18:0 alkylglycerol phosphate, a component expected on the basis of previous methanolysis and gas chromatographic analysis which revealed 1-stearylglycerol (Roberts et al., 1988). A structure which summarizes these data is presented in Fig. 2C and corresponds to a palmitoylinositol palmitoylated on the inositol group.

Additional information about the structure of the deamination product was obtained by similar FAB-MS and CID-MIKES approaches but with the sample prepared as the peracylated derivative. FAB-MS provided a series of clusters of molecular weight-related ions (M — H)~ whose masses were shifted to values consistent with replacement of four hydrogens with four acetyl groups (Fig. 3A). The shift is illustrated by the predominant molecular ion m/z 1303.7 (Δ = 1303.7 — 1135.7 = 168). This observation supported the premise of two endogenous substituents on inositol glycerol xylosides. The masses of three groups of ions at m/z 531.2/529.2, 405.2/403.2, and 373.2/371.2 were unaffected by peracylation and corresponded to the lipid-containing fragments discussed in Fig. 2. In contrast, three ions (m/z 665.2, 706.2, 973.5) exhibited the increase of 168 Da above their masses in Fig. 2B (m/z 497.2, 537.2, 805.5) that indicated addition of four acetyl groups. This structural interpretation is presented in Fig. 3B. One major ion at m/z 605.2 is less readily explained. This ion did not correspond to any ions observed in the spectrum of the inositol phospholipid prior to acetylation (Fig. 2A). Furthermore, it was not observed when the peracylated deamination product (M — H)~ ions were analyzed by CID-MIKES (data not shown), thus indicating that it was a distinct product of the acetylation reaction. The acetylation conditions here in fact were nearly as vigorous as those used previously to cleave alkylacylglycerols from the E°chu AChE anchor (Roberts et al., 1988), and thus a peracylated, palmitoylated inositol cyclic phosphate (Fig. 3C) arising from this cleavage would be expected. The molecular ion (M — H)~ (m/z 605.2 was

The m/z ratios observed in FAB or CID-MIKE spectra throughout this report agree with the theoretical values to within ±0.5 atomic mass unit. For example, the theoretical mass of the deamination product (M) with R1 = C18H37, R2 = C16H34, and R3 = C16H34 is 1138.9 compared with the observed value for (M — H)~ of 1137.7.

The masses of the daughter ions in the MIKE spectrum were rounded to the nearest integer.

The calculated masses of the structures proposed in Figs. 2, 3, and 5 and Table I were rounded to the next smaller integer.
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Explained by this structure. Analysis of the $m/z$ 605.2 ion by CID-MIKES analysis (data not shown) indicated a daughter ion ($m/z$ 366) consistent with a triacetylated inositol cyclic phosphate formed by the loss of a palmitoyl group from the structure shown in Fig. 3C.

To confirm further the structure in Fig. 2C, the purified deamination product was treated with ammonia-saturated methanol. Negative ion FAB-MS (data not shown) revealed molecular ions ($M - H)^{-}$, $m/z$ 897.7 and 899.7) with an ion distribution similar to that of the initial sample in Fig. 2A ($M - H)^{-}$, $m/z$ 1135.7 and 1137.7). This mass decrease of 238 Da in the molecular ion corresponded to selective palmitoyl deacylation and was also observed with all fragments previously characterized as containing inositol ($m/z$ 497.2, 573.2, and 805.5, Fig. 2A). Peracetylation of this methanolysis product and analysis by FAB-MS provided a characteristic molecular ion pattern with a mass increase of 210 Da which could be accounted for by replacement of five hydrogens with five acetyl groups (data not shown). Fragment ions at $m/z$ 735.5/737.7, previously characterized as products remaining after inositol elimination (Fig. 2C), were isobaric following both palmitoyl deacylation and reacetylation, as expected for reactions that involved only substituents on inositol. These observations verify our assertion that palmitic acid is esterified to inositol (also see Roberts et al., 1988).

*Analysis of Anchor Structures Isolated Following Proteolysis*—A dipeptide containing the glycoinositol phospholipid membrane anchor was prepared by papain proteolysis of $E^\text{nu}$ AChE radiomethylated with $[^1\text{C}]\text{HCHO}$ and NaCNBH$_3$ (Haas et al., 1986). The radiolabel permitted convenient identification of the anchor dipeptide product during purification by size exclusion chromatography on Sephadex LH-60 (Roberts and Rosenberry, 1986). Because of the fatty acyl heterogeneity and the extreme hydrophobicity of this proteolysis product, further purification was achieved by deacylation and reverse phase HPLC. A chromatogram of the base-treated product showed three radioactive peaks (data not shown) which corresponded to a nonretained fraction (8% of the...
applied 14C and a retained doublet of peaks that eluted in a mobile phase composition of about 40% 1-propanol. The peaks in this doublet contained 20 and 44% in the applied radioactivity, and the larger second peak was examined by both positive and negative ion FAB-MS. The positive ion spectrum (Fig. 4A) revealed two major ions at m/z 1730.9 and 1752.8 which were assigned as the protonated and sodiated molecular species, respectively. Analysis by negative ion FAB-MS also provided two major ions (Fig. 4B) at m/z 1728.9 and 1750.9 corresponding to the deprotonated and the dideprotonated, sodiated molecular ion, respectively. These results indicated the molecular weight of the papain proteolysis product to be 1729.9. In both analyses, each major ion was associated with a satellite ion 14 Da lower in mass that appeared to be consistent with incomplete reductive methylation. Unfortunately, neither spectrum exhibited fragmentation that could be used to establish a component sequence. This may be due to the presence of Triton X-100 which was detected in the positive ion spectrum by a characteristic ion pattern at 44-Da intervals (Fig. 4A). Spectral sequence information from FAB desorption analysis of biopolymers frequently is diminished by sample contaminants.

Detailed structural information was obtained from a more highly purified product of Pronase digestion of Ehu AChE that consisted of the glycoinositol phospholipid attached only to the C-terminal glycine residue. Greater care was taken to remove the Triton X-100 by pooling only eight-peak fractions from the Sephadex LH-60 column and by using a C8 instead of a C4 reverse phase HPLC column. Three radioactive HPLC peaks were obtained from the radiomethylated base-hydrolyzed Pronase proteolysis product. Their distribution was similar to that from the papain proteolysis product, with 18% of the 14C not retained and 21 and 44% in a doublet that eluted at a mobile phase composition of about 30% 1-propanol. A positive ion FAB spectrum of the major HPLC peak is presented in Fig. 5A, and the richer structural detail of the spectrum that arose from greater product purity than in Fig. 4 is obvious. The most abundant molecular ion at m/z 1593.9 (MH⁺) was accompanied by an associated sodium adduct ion (M + Na)⁺ that was 22 Da higher at m/z 1615.9. Incomplete methylation was again indicated by the detection of minor satellite ions 14 Da lower in mass. The molecular weight of the Pronase proteolysis product thus was 137 Da lower in mass than that generated by papain (Fig. 4A), consistent with amino acid analysis data that indicated only a C-terminal glycine residue and not the penultimate histidine residue found in the papain proteolysis product (Haas et al., 1986; data not shown). One other ion at m/z 1745.0 was of interest because the mass increment between it and the m/z 1593.9 ion of 151 Da was equal to an additional phosphoryl dimethylethanolamine group conjugated to the anchor. Although the low abundance of the m/z 1745.0 ion made it difficult to determine the location of this additional group, the detection of this ion suggested that a portion of the purified preparation contained an anchor with an additional phosphoryl dimethylethanolamine group (see "Discussion").

To interpret the spectra in Fig. 5, we propose the structure in Fig. 5C. This structure is suggested by the deamination product spectrum in Fig. 2A, the anchor components previously identified for Ehu AChE (Haas et al., 1986; Roberts et al., 1987, 1988) and previous reports of the anchor structure for trypanosome VSGs (Ferguson et al., 1985a, 1988; Schmitz...
et al., 1987). With the most abundant 18:0 alklyglycerol (Roberts et al., 1988), the proposed structure has a molecular mass of 1592.5, in good agreement with the predominant (MH)$^+$ ion at m/z 1593.9 in Fig. 5A. The anchor oligomer sequence could be deduced from mass spectral fragments generated as a consequence of labile bond rupture because the mass intervals between fragments were an indication of monomer array. As Fig. 5C indicates, most observed fragments included the phospholipid end of the structure. For carbohydrate oligomers, rupture can occur at the C$_1$-O glycosidic bond followed by transfer of a hydrogen to the glycosidic oxygen. Alternatively, pyranose ring rupture can occur with cleavage at the C$_3$-C$_2$ and C$_2$-O bonds with consequent retention of a formyl group on the glycosidic oxygen. The fragment that contains the reducing terminus generated by this cleavage is 28 Da larger than that produced by C$_1$-O rupture. Three ions arising from glycosidic bond cleavages were readily identified in the positive ion FAB mass spectra in Fig. 5A (m/z 1251.9, 1089.7, and 776.6) because they were accompanied by ions 28 Da larger in mass. The difference between the largest of these fragments and the molecular ion (1593.9 – 1251.9 = 342) corresponded to the glycerylaminomethylphosphorylhexose unit at the nonreducing terminus of the structure in Fig. 5C. Minor fragments which support this sequence were consistent with the loss of glucose only (m/z 1536.8) and of glycyamine (m/z 1493.5). The interval between the first pair of glycosidic bond cleavages (1251.9 – 1089.7 = 162) corresponded to a hexose residue without additional substituents, while that between the second pair of glycosidic bond cleavages (1089.7 – 776.6 = 313) was consistent with a hexose residue conjugated to phosphoryl dimethylsulfathalamine. Direct spectral evidence of this linkage was provided by a minor fragment ion at m/z 1522.8 that was 71 Da smaller than the (MH)$^+$ ion. This mass increment was consistent with cleavage of N,N-dimethylsulfathalamine with consequent loss of N,N-dimethylamine.

While identification of smaller fragments became difficult in the positive FAB spectrum in Fig. 5A, analysis by negative FAB-MS (Fig. 5B) both confirmed the fragments noted in Fig. 5A and yielded additional information on smaller fragments. A fourth ion arising from glycosidic bond cleavage (m/z 585.4) was identified by an accompanying ion 28 Da larger. The interval between the third pair of glycosidic bond cleavages in Fig. 5B (774.5 – 585.4 = 189) was consistent with a 2-deoxy-2-dimethylaminohexose residue, a component expected from a previous observation of radiomethylated glucosamine (Haas et al., 1986). The fragment ion at m/z 423.2 was 162 Da smaller than the m/z 585.4 ion, but the interval does not appear to indicate loss of a pyranose residue because the m/z 423.2 ion was not accompanied by an ion 28 Da larger. This interval was consistent with an inositol adjacent to the glucosamine, and the m/z 423.2 fragment was precisely the 18:0 alklyglycerol phosphate deduced from the deamination product spectrum in Fig. 2A.

To corroborate the positions of reductively methylated residues proposed in Fig. 5C, a radiomethylated Pronase proteolysis product was prepared using NaCNBD$_3$ as the reducing agent instead of NaCNBH$_3$. Under these conditions one deuterium atom was incorporated for each methyl group introduced to give a mass increase of 2 Da/methylated residue. Positive and negative ion FAB mass spectra of the Pronase proteolysis product methylated with deuterium reduction were acquired, and the incremental mass differences for the major fragments were calculated and summarized in Table I. The ion at m/z 1743 contained six methyl groups consistent with two N,N-dimethylsulfathalamine residues as suggested above. A mass difference of 4 Da was observed for the m/z 1592 Pronase proteolysis product molecular ion and most of the progressively smaller distal sequence fragments (Fig. 5C) until loss of the hexose linked to phosphorylaminomethylphosphorylhexose (m/z 803). One exception was the m/z 1521 ion which was proposed above to represent loss of dimethylsulfathalamine from the parent molecule, and as expected this fragment contained only two methyl groups. Two ions, m/z 803 and 775, contained only two methyl groups, as predicted for the fragments containing glucosamine as the only pyranose (Fig. 5C). Following loss of this hexosamine residue (m/z 614) the remaining cleavages in the sequence are isobaric, again con-
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**FIG. 5.** FAB-MS analysis of the base-hydrolyzed Pronase proteolysis product. Panel A, positive ion FAB mass spectrum. For \( m/z \) values between 1000 and 1550 (arrows) the relative abundances were amplified by a factor of 2. Panel B, negative ion FAB mass spectrum. For \( m/z \) values between 750 and 1050 (arrows) the relative abundances were amplified by a factor of 2, and for \( m/z \) values greater than 1050, the amplification was by a factor of 10. The triethanolamine matrix gave rise to an ion at \( m/z \) 446.3 (*). Panel C, proposed structure of the Pronase proteolysis product summarizing results from Panels A and B.

consistent with Fig. 5C. This pattern supports the structure proposed in Fig. 5C, including the adjacent arrangement of the two pyranose rings with reductively methylated residues.

**DISCUSSION**

Although most of the FAB mass spectra in the literature were obtained in the positive ion mode, molecular ions from the \( E^{\text{nu}} \) AChE nitrous acid deamination product and its derivatives here were observed only in negative ion FAB mass spectra. The phosphate group apparently was responsible for anion formation in nearly all of the fragments arising from the deamination product. In contrast, molecular ions were observed for the papain and Pronase proteolysis products in both the positive and negative ion mode. Numerous positive and negative fragment ions were produced from the Pronase product probably resulting from charge stabilization by the anionic phosphate groups and cationic tertiary amines distributed throughout the molecule. The use of CID-MIKES was a powerful adjunct to the fragmentation produced by FAB ionization of the deamination protocol. It provided both evidence that a given fragment ion originated from the molecular ion and confirmation of its structure.

The chemical structure of the glycoinositol phospholipid
one deuterium atom was incorporated per methyl group and two
determined by comparing the masses of FAB ions in positive and
negative spectra. When NaCNBD₃ was used as the reducing agent,
formaldehyde and reduction with either NaCNBH₃ or NaCNBD₃ was
the anchors of Thy-1 (Fatemi et al., 1987). The unblock amino
group also has been found associated with
uent may be common to many glycoinositol phospholipid
ctions of hexose or inositol linkages. FAB-MS of the Eh" AChE
experiments are necessary to establish the stoichiometry and
anchor of Trypanosoma brucei VSGs has recently been ex-
aminined independently by two research groups who used several
techniques including nuclear magnetic resonance spec-
troscopy, FAB-MS, permethylation analysis, and exoglycoxi-
dase digestion (Schmitz et al., 1987, VSG MITat 1.6; Ferguson et al., 1988, VSG MITat 1.4). A schematic representation of the complete structure of the VSG membrane anchor based on one of these reports (Ferguson et al., 1988) is shown in Fig. 6 along with a summary of the E₈⁺ AChE anchor structure derived from data presented here and in the preceding paper (Roberts et al., 1988). Both the VSG and AChE anchors have a core glycan consisting of 3 hexose residues linked to etha-
nolamine at the protein C terminus through a phosphate
group and to an inositol phospholipid through a glucosamine
residue. The three hexoses in the core glycan of VSG anchors
are mannose. Preliminary data indicate mannose to be the
only neutral hexose in the E₈⁺ AChE anchor, but additional
experiments are necessary to establish the stoichiometry and
to identify potential mannose phosphates. Complete hexose
linkages and anemic configurations have been reported for the T. brucei VSG MITat 1.4 anchor (Ferguson et al., 1988). Unfortunately, FAB-MS and CID-MIKES analyses of the E₈⁺
AChE anchor revealed no fragments that indicate the posi-
tions of hexose or inositol linkages. FAB-MS data of the E₈⁺ AChE
protonate proteolysis product did clearly indicate that all three
hexoses and the glucosamine are joined by glycosidic linkages,
and compositional analysis demonstrated myo-inositol 1-
phosphate in the E₈⁺ AChE anchor (Roberts et al., 1988).

Two major differences between the two anchors in Fig. 6 are evident. First, different substituents branch from the hexose adjacent to the glucosamine. In VSG MITat 1.4 this substituent is heterogenous and consists of a branched oligo-
saccharide of 2-4 galactose units. Even greater heterogeneity
(0-8 galactose units) is observed among VSG variants
(Holder, 1985). In contrast, the AChE anchor substituent is an
additional phosphorylthanolamine moiety. This substitu-
ent may be common to many glycoinositol phospholipid
anchors on mammalian proteins, since ethanolamine with an
unblocked amino group also has been found associated with
the anchors of Thy-1 (Fatemi et al., 1987), decay accelerating
factor (Medof et al., 1986), and Drosophila (Gnagey et al.,
1987) and bovine erythrocyte AChE (Roberts et al., 1987).

Evidence of an additional phosphorylthanolamine residue on some of the E₈⁺ AChE anchors is suggested by a less abundant (MH)⁺ ion at m/z 1745, but no information about
its location was obtained in these experiments. The presence of some E₈⁺ AChE anchors with two terminal phosphorylid-
methylethanolamine residues also is supported by the overall
stoichiometry of 1.3 mol of ethanolamine with free amino
groups per mol of AChE anchor (Haas et al., 1986). A second
difference between the VSG and AChE anchors involves their
lipid components. The VSG anchor contains exclusively di-
myristoylphosphatidylcholine (Ferguson et al., 1985a, 1985b).
The AChE anchor is a plasmanylinositol containing alkyl-
acylglycerols that have predominantly 18:0 alkyl and 22:4
and 22:5 acyl groups (Roberts et al., 1988). Perhaps most
intriguing, a palmitoyl group acylates the inositol ring of
this plasmanylinositol. Although Brennan (1968) reported
some evidence for a triacylated phosphatidylcholine in Corynebacterium xerosis, the FAB-MS data in Figs. 2 and 3 provide, to our knowlaged, the first documentation of fatty
acid acylation of inositol in any system and complement fatty
acid composition data in the accompanying paper (Roberts et al.,
1988) which support this conclusion. FAB-MS data also
dicate that treatment with ammonia-saturated methanol
preferentially deacylates this palmitoyl group from the plas-
manylinositol deamination product. This finding was used to
demonstrate that palmitoylation of the inositol is solely re-
ponsible for the resistance of the palmitoylated plasmanyli-
ositol to phosphatidylinositol-specific phospholipase C from
S. aureus (Roberts et al., 1988).

It is noteworthy that the two reported VSG anchor struc-
tures differ in a fundamental respect. In contrast to the linear
orientation of the 3 core mannose residues proposed for the
anchor of VSG MITat 1.4 (Ferguson et al., 1988), data from the
anchor of VSG MITat 1.6 was interpreted as indicating a brancned three-mannose core, with two mannoses linked α1–
6 and α1–3 to the distal mannose residue (Schmitz et al.,
1987). The data we obtained by FAB-MS analysis of the base-
hydrolyzed Pronase product support a core glycan with a linear
structure (Fig. 6) like that described for VSG MITat 1.4 (Ferguson et al., 1988). A structure with the branched
glycan proposed for the anchor of VSG MITat 1.6 when applied to the E₈⁺ AChE anchor would predict a major pair of
fragment ions at m/z 1429/1458 arising from cleavages of the
glycosidic linkage of the putative terminal hexose in the base-
hydrolyzed Pronase proteolysis product, neither of which was

\begin{table}
\centering
\caption{Mass Spectrometric Analysis of the E₈⁺ AChE Membrane Anchor}
\begin{tabular}{|c|c|}
\hline
Fragment mass & Mass shift \\
\hline
586 & 0 \\
614 & 0 \\
775 & 2 \\
803 & 2 \\
1088 & 4 \\
1116 & 4 \\
1250 & 4 \\
1278 & 4 \\
1492 & 4 \\
1521 & 2 \\
1592 & 4 \\
1743 & 6 \\
\hline
\end{tabular}
\end{table}
observed. The branched structure also would not account for the major fragment ions at m/z 1087/1116 except in the unlikely case that these ions reflected bond rupture at two locations in the parent molecule.

The structure in Fig. 6 reflects the predominant component in the major HPLC peak from both the papain and the Pronase digestion products of $E_{nu}$ AChE. The two minor HPLC peaks from the Pronase digest both contained glycine as well as radiomethylated ethanolamine and glucosamine. Attempts to obtain FAB-MS data on the retained minor peak were unsuccessful. The less hydrophobic nonretained minor peak may have lost both fatty acids and alkylglycerols during base hydrolysis, but its structure was not pursued because of the presence of nonretained contaminants.

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Note Added in Proof—A structure for the rat brain Thy-1 glycoinositol phospholipid anchor has recently been reported (Homans, S. W., Ferguson, M. A. J., Dwek, R. A., Rademacher, T. W., Anand, R., and Williams, A. F. (1988) Nature 333, 269-272) and illustrates further heterogeneities in anchor structures.

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