Glycan Components in the Glycoinositol Phospholipid Anchor of Human Erythrocyte Acetylcholinesterase

NOVEL FRAGMENTS PRODUCED BY TRIFLUOROACETIC ACID*

(Received for publication, January 30, 1992)

Mark A. Deeg, Dawn R. Humphrey, Shun Hua Yang, Todd R. Ferguson, Vernon N. Reinhold, and Terrone L. Rosenberry‡

From the Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106 and the Division of Biological Sciences, Harvard School of Public Health, Boston, Massachusetts 02115

Inositol glycans were prepared from reductively radioiodinated human erythrocyte acetylcholinesterase by sequential treatment with Proteinase K, methanolic KOH, and phosphatidylinositol-specific phospholipase C. Four glycans denoted α-δ were resolved by anion exchange high performance liquid chromatography (HPLC). Each glycan was subjected to hydrolysis in 4 M trifluoroacetic acid, and their hexose and hexose phosphate compositions were determined by anion exchange HPLC. The predominant glycan α showed a relative stoichiometry of 2 mannoses, 1 mannose 6-phosphate, 1 radiomethylated glucosamine, 1 radiomethylated ethanolamine, and 1 inositol. In contrast, the stoichiometry of glycan β was 1 mannose, 2 mannose 6-phosphates, 1 radiomethylated glucosamine, 2 radiomethylated ethanolamines, and 1 inositol. Glycans α and β were analyzed by electrospray ionization mass-spectrometry, and respective parent ions of m/z 1266 and 1417 were observed. The fragmentation pattern produced by collision-induced dissociation mass spectrometry of these parent ions was consistent with a common linear core glycan sequence prior to radiomethylation of ethanolamine-phosphate-mannose - mannose - mannose - glucosamine - inositol. Glycan α contained a single additional radiomethylated phosphoethanolamine branching from the mannose adjacent to glucosamine, whereas glycan β contained two additional radiomethylated phosphoethanolamines, one branching from each of the mannoses nearest to glucosamine. Trifluoroacetic acid hydrolysis did not cleave within the N,N-dimethylglucosamine-inositol-phosphate moiety in these glycans, and this component was resolved by anion exchange HPLC and structurally confirmed by mass spectrometry. Dephosphorylation of this component by treatment with 50% HF produced N,N-dimethylglucosamine-inositol, and this conjugate was shown to have a characteristic elution time on cation exchange chromatography in an amino acid analyzer. Both of these fragments involving an intact radiomethylated glucosamine-inositol bond are proposed as new diagnostic indicators in the search for minor glycoinositol phospholipids in cells and tissues.

The membrane association of many integral membrane proteins is mediated at least in part by covalently attached lipid groups. Among intracellular proteins, the attachments can involve acylation of protein residues by the fatty acids myristate or palmitate (Sefton and Buss, 1987) or the prenylation of cysteine residues by farnesyl or geranylgeranyl groups (Maltese, 1990). In contrast, more than 50 identified extracellular membrane proteins are anchored exclusively by a glycoinositol phospholipid (GPI) linked covalently to the protein C terminus (see reviews by Ferguson and Williams (1988), Rosenberry et al. (1989), and Cross (1990)). GPI anchors may be considered as a reasonably well defined class of structures because important elements are conserved over the wide range of phylogeny from protozoan parasites to mammals. Complete GPI structural determination involves a variety of analytical approaches to deduce the core oligosaccharide and inositol sequence and linkage positions, the lipid composition and structure, and the location and nature of all branching groups. Detailed information is available on the GPI anchors of four proteins, of which three are outlined in Fig. 1. The first complete structure was obtained for variant surface glycoprotein (VSG), an abundant class of surface proteins on Trypanosoma brucei (Ferguson et al., 1988). This anchor contains a linear core glycan sequence of ethanolamine-P0-6Manα1-2Manα1-6Manα1-4GlcNH2α1-6-myoinositol-1-P0. This core sequence is completely conserved in the GPIs of rat Thy-1 (Homans et al., 1988) and Leishmania major promastigote surface protease (Schneider et al., 1990), and previously reported structural information on the GPI of human erythrocyte (Eβ) acetylcholinesterase (AChE) is entirely consistent with it (Roberts et al., 1988b). A complicating factor in these analyses is the heterogeneity apparent with several GPI anchors. One source of GPI heterogeneity is the lipid composition. The VSG anchor contains only dimethylpropionylphosphatidylinositol (Ferguson et al., 1985), whereas GPIs of Leishmania promastigote surface protease and many mammalian GPI-anchored proteins, including those on erythrocyte

*The abbreviations used are: GPI, glycoinositol phospholipid; VSG, trypanosome variant surface glycoprotein; Eβ, human erythrocyte; AChE, acetylcholinesterase; PIPLC, phosphatidylinositol-specific phospholipase C; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; FAB, fast atom bombardment; ESI, electrospray ionization; CID, collision-induced dissociation; PAD, pulsed amperometric detector; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Etn(NMe)2, N,N-dimethyl ethanolamine; Glc(NMe)2, N, N-dimethyl glucosamine; Glc(NMe)3-InsP, N,N-dimethyl glucosamine-inositol; Man-Glc(NMe)3-InsP, mannose-N,N-dimethyl glucosamine-inositol-phosphate.

1The abbreviations used are: GPI, glycoinositol phospholipid; VSG, trypanosome variant surface glycoprotein; Eβ, human erythrocyte; AChE, acetylcholinesterase; PIPLC, phosphatidylinositol-specific phospholipase C; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; FAB, fast atom bombardment; ESI, electrospray ionization; CID, collision-induced dissociation; PAD, pulsed amperometric detector; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Etn(NMe)2, N,N-dimethyl ethanolamine; Glc(NMe)2, N, N-dimethyl glucosamine; Glc(NMe)3-InsP, N,N-dimethyl glucosamine-inositol; Man-Glc(NMe)3-InsP, mannose-N,N-dimethyl glucosamine-inositol-phosphate.

18573
structures yet known to deviate from the core glycan sequence noted above are found in *Leishmania*, where both the GPI anchor of a lipophosphoglycan (Turco et al., 1989) and free GPI glycolipids (McConville et al., 1990) retain only the mannose-glucosamine-inositol-phosphate portion of the core but replace other residues toward the nonreducing terminus of the core with sugars that include galactose. Although the functional significance of this GPI heterogeneity is unclear, it may reflect subtle differences in interactions with other membrane components or in targeting to specific cell organelles.

The glycan structures of VSG and Thy-1 in Fig. 1 were established by a combination of two-dimensional proton NMR, GC-MS analysis of partially permethylated alditol acetates, and size analysis on Bio-Gel P4 in conjunction with HF and exoglycosidase cleavage. Smaller amounts of available Eh" AChE anchor have precluded NMR analysis, and the structure in Fig. 1 was deduced by lipid composition analyses and fast atom bombardment-mass spectrometry (FAB-MS). These procedures do not identify the hexose residues or the linkage positions in the AChE anchor glycan, and this report provides new information on these points. In addition, we continue to apply reductive radiomethylation to label free amine groups in the GlcN and ethanolamine residues of the AChE GPI anchor. Novel radiolabeled fragments produced by trifluoroacetic acid and HF cleavage of the radiomethylated GPI anchor are characterized. These procedures offer new criteria for the identification of GPls in tissue extracts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bacillus thuringiensis PIPLC was purified from the culture medium of a Bacillus subtilis strain that had been transformed with the gene for *B. thuringiensis* PIPLC (Henner et al., 1988; kindly provided by Dr. Martin Low, Columbia University, New York). Proteinase K was from Gibco-BRL. Glc-1-P and fructose 1,6-diphosphate (barium salt) were from ICN (Costa Mesa, CA), whereas other hexose, hexose phosphate, and inositol analytical standards were from Sigma.

**Protein Purification and Radiolabeling**—AChE was extracted from outdated human erythrocytes with Triton X-100 and purified by affinity chromatography on acridium resin as described in Rosenberry and Scoggin (1984), except that a more extensive resin wash was employed before enzyme elution (Gnagey et al., 1987). The purified Eh" AChE (5–200 nmol) in 0.1% Triton X-100 was concentrated 10–25-fold in a SpeedVac concentrator (Savant Instruments), diluted to a stock concentration, and further purified Eh" AChE anchor was reductively radiomethylated with un- and re- radiomethylated 10–20 mM ["C]HCHO (ICN, either stock 56 mCi/mmol or 0.1–10 mCi/mmol by dilution with unlabeled HCHO) and 50–65 mM NaCNBH3 (Haas et al., 1986), and dialyzed extensively against 10 mM HEPES, 0.05% Triton X-100 (pH 7.0–7.5). The methyl group specific activity deriving from the stock ["C]HCHO was estimated to be 96 cpm/pmol (Haas and Rosenberry, 1985; Haas et al., 1986), and methyl group specific activities for GPI anchor fragments radiomethylated with diluted stocks were calculated from the measured ratio of incorporated label to inositol relative to the ratio for the corresponding fragments radiomethylated with undiluted stock.

**Isolation of Intact Inositol Glucans from Eh" AChE**—To the dialyzed concentrate of radiomethylated Eh" AChE and Triton X-100 in 10 mM HEPES was added Proteinase K (to 5 mg/ml) and CaCl2 (to 2 mM), and the mixture was incubated at 50 °C for 10–22 h. After addition of sodium dodecyl sulfate (to 1%) and more Proteinase K (additional 1 mg/ml), the digestion was continued at 50 °C for 5–17 h. The digestion mixture was applied to a Sephacryl S-200 column (1.5 × 80 cm) equilibrated in 10 mM HEPES (pH 7.5), 1 mM Na2SO4, and 0.05% Triton X-100. The fragment containing the C-terminal glycine residue linked to the GPI is the only fragment to associate with detergent micelles and elutes in the first peak of radioactivity at an elution volume of 0.55 relative to the solvent marker (Roberts et al., 1985; Haas et al., 1986).

---

3. J. A. Kral, P. J. Thomas, Q. W. Yang and T. L. Rosenberry, procedure to be published.
Glycan Components in the Membrane Anchor of E\textsuperscript{th} AChE

RESULTS

The structure of the E\textsuperscript{th} AchE anchor in Fig. 1 was deduced from previous work in our laboratory that involved PAB-MS analysis of the radiomethylated GPI fragments obtained after complete pronase digestion and deacylation with base (Roberts et al., 1988b). Individual hexose residues in the glycan and their linkage positions could not be determined by this procedure. To isolate homogeneous E\textsuperscript{th} AchE anchor glycans for further analysis, the proteolyzed and deacylated GPI fragments were digested with PIPLC to remove the alkylglycerol residue and chromatographed on anion exchange HPLC as outlined in Fig. 2. Four peaks identified by PAD and containing radiolabel were obtained and designated α, β, γ, and δ.

Individual hexose components of the predominant glycan α were determined by hydrolysis with trifluoroacetic acid and anion exchange HPLC as shown in Fig. 3. The PAD trace in Fig. 3D shows three components, Glc, Man, and Man-6-P, that align with the standards in Fig. 3A. However, a similar amount of Glc was also present in the trifluoroacetic acid hydrolysate blank in Fig. 3C. In addition to these three components, four other peaks denoted a–d are apparent in the PAD trace in Fig. 3D. Fractions corresponding to peaks a, c, and d contained components labeled by radiomethylation as shown in Fig. 3E. Furthermore, GC-MS analysis indicated that virtually all of the inositol was in peaks c and d (Table I). Peak b was not identified.

We have shown previously that the reductive radiomethylation procedure employed here converts free amine groups in AChE to their dimethylamine derivatives, and two components containing free amines, ethanolamine and GlcN, were identified in 6 N HCl hydrolysates of the isolated GPI anchor by cation exchange chromatography on an amino acid analyzer (Haas et al., 1987). This is illustrated in Fig. 4A, where the hydrolysates of EthN(Me)\textsubscript{2} AChE GPI fragments isolated after Proteinase K digestion are compared at various hydrolysis times. After 16 h, hydrolysates contained about equal amounts of radiomethylated ethanolamine and GlcN components, as observed previously (Haas et al., 1986) and as predicted from Fig. 1. At shorter hydrolysis times, the recovery of labeled EthN(Me)\textsubscript{2} remained constant, but the recovery of labeled

Haynes, R. C., and R. H. Vold (1961). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 44, 609-619.

Haynes, R. C., and R. H. Vold (1962). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 45, 77-95.

Heath, R. A. (1977). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 859-871.

Heath, R. A. (1978). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 873-883.

Heath, R. A. (1979). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 885-892.

Heath, R. A. (1980). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 893-900.

Heath, R. A. (1981). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 901-908.

Heath, R. A. (1982). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 909-915.

Heath, R. A. (1983). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 917-923.

Heath, R. A. (1984). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 925-931.

Heath, R. A. (1985). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 933-939.

Heath, R. A. (1986). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 941-947.

Heath, R. A. (1987). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 949-955.

Heath, R. A. (1988). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 957-963.

Heath, R. A. (1989). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 965-971.

Heath, R. A. (1990). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 973-979.

Heath, R. A. (1991). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 981-987.

Heath, R. A. (1992). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 989-995.

Heath, R. A. (1993). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 997-1003.

Heath, R. A. (1994). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1005-1011.

Heath, R. A. (1995). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1013-1019.

Heath, R. A. (1996). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1021-1027.

Heath, R. A. (1997). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1029-1035.

Heath, R. A. (1998). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1037-1043.

Heath, R. A. (1999). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1045-1051.

Heath, R. A. (2000). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1053-1059.

Heath, R. A. (2001). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1061-1067.

Heath, R. A. (2002). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1069-1075.

Heath, R. A. (2003). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1077-1083.

Heath, R. A. (2004). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1085-1091.

Heath, R. A. (2005). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1093-1099.

Heath, R. A. (2006). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1101-1107.

Heath, R. A. (2007). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1109-1115.

Heath, R. A. (2008). "The role of the potassium ion in the action of the enzyme acetylcholinesterase." J. Gen. Physiol. 69, 1117-1123.
phosphate ester bonds and subjected to cation exchange chromatography. The sample (300 μl) was fractionated by Dionex anion exchange HPLC with the following gradient elution protocol: 0-5 min, isocratic 100 mM NaOH plus 150 mM sodium acetate; 5-65 min, linear gradient of 100 mM NaOH plus 150 mM sodium acetate to 100 mM NaOH plus 310 mM sodium acetate. In this run, the HPLC effluent was connected to the PAD and anion suppressor noted under “Experimental Procedures.” Four peaks identified both by PAD and anion suppressor were tentatively defined as GPI fragments in order of elution. Two percent of each 1.0-ml fraction was taken for scintillation counting in a Beaker. Recovery of radioactivity was 68 ± 6% (S.D., four independent preparations), of which 59 ± 4% (S.D.) was in α and 17 ± 2% (S.D.) was in γ.

GlcN(Me)₂, and its hydrolysis product X₆ decreased. This decrease was accompanied by an increase in two early peaks, one at 8 min and the second at 27 min (denoted X₀ in Haas et al., 1986). These observations suggested that GlcN(Me)₂ remained partially linked to other components under less rigorous acid hydrolysis conditions. This suggestion was confirmed by further analysis of the radiolabeled peaks in Fig. 3E by cation exchange chromatography. The label in peak α consisted exclusively of EthN(Me)₂ with or without further acid hydrolysis, whereas the label in peaks c and d corresponded only to GlcN(Me)₂ components after further hydrolysis in 6 N HCl (Table I). Without further hydrolysis, peaks c and d were not retained on cation exchange chromatography (data not shown), as expected from their elution in the region of phosphate-containing standards in Fig. 3. Given the presence of inositol in peaks c and d and the structures in Fig. 1, it appeared likely that these peaks contained a trifluoroacetic acid-resistant core consisting of GlcN(Me)₂-Inos-P.

To establish this trifluoroacetic acid-resistant core structure, peak c in Fig. 3E was treated with 50% HF to cleave phosphate ester bonds and subjected to cation exchange chromatography. A peak comigrating with X₆ was observed (Fig. 3).

FIG. 2. Anion exchange chromatography of GPI fragments obtained from radiomethylated E₇₈ AChE following complete proteolysis and PIPLC cleavage. Proteinase K and PIPLC digestions were conducted on E₇₈ AChE radiomethylated with [³⁵S]methionine as outlined under “Experimental Procedures.” The sample (300 μl) was fractionated by Dionex anion exchange HPLC with the following gradient elution protocol: 0-5 min, isocratic 100 mM NaOH plus 150 mM sodium acetate; 5-65 min, linear gradient of 100 mM NaOH plus 150 mM sodium acetate to 100 mM NaOH plus 310 mM sodium acetate. In this run, the HPLC effluent was connected to the PAD and anion suppressor noted under “Experimental Procedures.” Four peaks identified both by PAD and anion suppressor were tentatively defined as GPI fragments in order of elution. Two percent of each 1.0-ml fraction was taken for scintillation counting in a Beaker. Recovery of radioactivity was 68 ± 6% (S.D., four independent preparations), of which 59 ± 4% (S.D.) was in α and 17 ± 2% (S.D.) was in γ.

FIG. 3. Anion exchange chromatographic identification of hexose, hexose phosphate, and radiomethylated components obtained by 4 M trifluoroacetic acid hydrolysis of glycan α prepared as in Fig. 2. Conditions for trifluoroacetic acid hydrolysis and gradient elution of the Dionex Carbopac column are given under “Experimental Procedures.” Panels A–D, PAD traces. Panel A standards: 1, fucose; 2, GalN; 3, GlcN; 4, Gal; 5, Glc; 6, Man; 7, myo-inositol 1,2-cyclic phosphate; 8, myo-inositol 2-phosphate; 9, Glc-1-P; 10, Man-6-P; 11, fructose 1,6-diphosphate. Increases in the background PAD signal reflect changes in the acetate concentration in the gradient. Panel B, glycan α isolated as in Fig. 2 and desalted on Bio-Gel P2. Panel C, 4 M trifluoroacetic acid hydrolysate of a blank composed of nonradioactive Bio-Gel P2 desalting column fractions adjacent to α. Panel D, 4 M trifluoroacetic acid hydrolysate of Bio-Gel P2-desalted column fractions containing α. Glc is denoted glu in panels C and D. Panel E, radioactivity corresponding to 50% of each 1.0-ml fraction collected from the run in panel D. The α samples analyzed in panels B and D contained 2 nmol of myo-inositol as determined by independent GC-MS analysis. Recovery of radioactivity was 79 ± 5% and, of inositol, 96 ± 10% (S.D., n = 5) from chromatographic runs of trifluoroacetic acid hydrolysates of α samples.
the only 6-hydroxyl group available in the core glycan in Fig. EthN(Me)2 phosphate linked to a Man 6-hydroxyl group, and inositol. These observations are consistent with an extra

$$\frac{Q}{3},$$
charged, and the composition data support this point. Glycan ions with each residue of inositol are in excellent agreement with the

$$\text{HCl}$$ and cation exchange chromatography (Fig. 4).

TABLE I

| Trifluoroacetic acid | Inositol | GlcN(Me)2 | EthN(Me)2 | % of total recovery
|---------------------|---------|-----------|-----------|------------------
| a                   | 5       | 0         | 100       |                  
| b                   | <2      | 0         | 0         |                  
| c                   | 63      | 66        | 0         |                  
| d                   | 32      | 34        | 0         |                  

* GC-MS analysis of 10-fractiopn pools from the chromatographic run in Fig. 3E revealed significant amounts of inositol only in fractions containing a, c, and d. GlcN(Me)2 and EthN(Me)2 recoveries were calculated from the distribution of total radioactivity among the three labeled fragment pools in Fig. 3E and the amine contents of each pool determined by 16-h hydrolysis in 6 N HCl and cation exchange chromatography (Fig. 4).

4B) that contained equal amounts of labeled GlcN and inositol. Cleavage of glycan α first with HF and then with trifluoroacetic acid gave the same labeled product X, (Fig. 4C). Further analysis of peak c by ESI-MS revealed two prominent phosphorylated species, a major m/z 450.2 ion that corresponded precisely to GlcN(Me)2-Inos-P and a minor m/z 532.3 ion consistent with Man-GlcN(Me)2-Inos-P. Peak c in Fig. 3D appears to be a fused peak, consistent with a mixture of these two components. However, the MS and cation exchange chromatography data confirm that the predominant component in peak c is trifluoroacetic acid-resistant GlcN(Me)2-Inos-P and indicate that its dephosphorylated derivative X, is GlcN(Me)2-Inos. These conclusions are summarized in Table II.

Quantitation of glycan α components by trifluoroacetic acid hydrolysis and anion exchange HPLC as in Fig. 3 depended on the hydrolysis conditions. As the trifluoroacetic acid concentration was increased from 1.0 to 4.0 M, recoveries of Man and Man-6-P increased progressively and the ratio of peak c to peak d also increased (Table II). The late elution position of peak d suggests that phosphate groups are retained in addition to that in the GlcN(Me)2-inosine F core, and the structures in Fig. 1 indicate that this can occur only if these fragments also retain Man groups. Thus, it is likely that initial trifluoroacetic acid fragments in peak d are broken down to Man, Man-6-P, and peak c at higher trifluoroacetic acid concentrations. Since peak d is still detected even after 4 M trifluoroacetic acid hydrolysis (Fig. 3E), the stoichiometries of Man and Man-6-P in Table III are slight underestimates. Despite this limitation, the observed stoichiometries in glycan α of nearly 2 residues of Man, 1 residue of Man-6-P, 1 residue of GlcN(Me)2, and 1 residue of EthN(Me)2, for each residue of inositol are in excellent agreement with the GPI anchor structures in Fig. 1.

Composition data for the other GPI fragments obtained from Ema AChE in Fig. 2 are compared with that for glycan α in Table IV. Glycan β was eluted later than α during the HPLC run in Fig. 2, suggesting that it was more negatively charged, and the composition data support this point. Glycan β differs from α in containing 2 residues of Man-6-P, 1 residue of Man, and 2 residues of EthN(Me)2 for each residue of inositol. These observations are consistent with an extra EthN(Me)2 phosphate linked to a Man 6-hydroxyl group, and the only 6-hydroxyl group available in the core glycan in Fig. 1 is on Man2.º To confirm these structures for glycan α and β, samples were analyzed by ESI-MS. Predominant (MH+2)2 ions with m/z of 634.1 and 709.7 were observed for α and β, respectively, and the CID-MS spectra derived from these molecular ions are shown in Fig. 5. The major CID fragments are accounted for by rupture of Man glycosidic bonds or phosphodiester linkages as indicated. The mass difference between glycans α and β (1417 - 1266 = 151) is accounted for by the additional EthN(Me)2 phosphate linked to Man2. Glycan γ had a composition similar to α (data not shown), but it was such a minor component that additional character-
Phosphate linkage at the 6-position of one Man, presumably Furthermore, all of the isolated AChE glycans account for some of the charged parent ion at m/z (709)\(^+\). Two product fragment ions at m/z 538 and 660 were observed to be doubly charged. The m/z 538 ion is the doubly charged counterpart of the singly charged ion at m/z 1076 fragment. The m/z 660 ion is consistent with loss of a phosphate from the parent ion, which would represent a decrement of 49 Da for these doubly charged ions. The fragmentation scheme is indicated at the top.

**Fig. 5.** Panel A, collision spectrum (MS-CID-MS) of glycan α obtained by selecting the doubly charged parent ion at m/z (634)\(^+\). All fragments were observed to be singly charged, and they were produced as indicated in the scheme at the top. Panel B, collision spectrum (MS-CID-MS) of glycan β obtained by selecting the doubly charged parent ion at m/z (709)\(^+\). Two product fragment ions at m/z 538 and 660 were observed to be doubly charged. The m/z 538 ion is the doubly charged counterpart of the singly charged ion at m/z 1076 fragment. The m/z 660 ion is consistent with loss of a phosphate from the parent ion, which would represent a decrement of 49 Da for these doubly charged ions. The fragmentation scheme is indicated at the top.
**Glycan Components in the Membrane Anchor of β^th ACHE**

Man^3^2 These features are consistent with those previously reported for VSG and Thy-1 in Fig. 1. The ACHE glycans also all contain phosphoethanolamines with amine groups available for radiomethylation, and ESI-MS analysis of the radiomethylated α and β glycans showed EthN(Me)_2 phosphate located on Man1. A similar phosphoethanolamine is linked to the 2-position of the first Man in Thy-1. Glycan β, involved in 10–20% of the ACHE GPI anchors, includes a modification not previously documented in any GPI anchor: a second phosphoethanolamine with a phosphodiester linkage to the 6-position of Man2. The linkage here is unequivocal, as trifluoroacetic acid hydrolysis demonstrated an increase of one Man-6-P and a decrease of one Man in α relative to α. A glycan such as β with two methylated ethanolamine phosphates was inferred from the initial FAB-MS studies (Roberts et al., 1988b), but the residue to which the additional phosphoethanolamine group was attached could not be deduced.

Although the position of phosphate linkage to Man1 in the ACHE glycans cannot be established from the available data, several observations are pertinent. First, composition data on glycan β following 4 m trifluoroacetic acid hydrolysis reveal Man residues (Table IV). Since control hydrolyses indicated that only about 6% of Mar-6-P is degraded to Man during trifluoroacetic acid hydrolysis, this Man must have derived from a Man phosphate residue in β with a linkage more labile to trifluoroacetic acid than Man-6-P. Second, ESI-MS analysis of trifluoroacetic acid fragments in peak c detected a Man-GlcN(Me)_2-Inos-P fragment apparently devoid of a Man phosphate linkage, suggesting that the phosphate linkage to Man1 in the ACHE glycans is more labile to trifluoroacetic acid than Man-6-P. Finally, unidentified peak b in Fig. 3D is eluted in the hexose phosphate region but contains no inositol or radiomethylated amine. This peak has the characteristics of a trifluoroacetic acid hydrolysis intermediate, as it is greater in 1 m trifluoroacetic acid than in 4 m trifluoroacetic acid. These are characteristics expected of a partially labile mannose phosphate that could represent Man1 in the ACHE glycans. Unfortunately, appropriate mannose phosphate standards are not available to test this assignment.

Completion of the β^th ACHE GPI anchor structure involves assignment of the core glycan linkages by permethylation and two-dimensional NMR analyses, techniques that require more purified glycan than that used in these studies. However, a major objective of this report is to introduce a new set of GPI fragments that can be used in identifying and characterizing GPI structures. The elegant studies of Ferguson and his colleagues (Ferguson et al., 1988; Ferguson, 1992) have provided several procedures that are diagnostic of GPI fragments, but most of the analyses involve complete dephosphorylation in 50% HF and characterization of the resulting neutral glycans. Direct fragmentation of GPls by trifluoroacetic acid is shown in this report to provide a complementary approach that allows simultaneous Dionex ion exchange analysis of neutral hexoses, hexose monophosphates, and the glucoamine-inositol-phosphate core that many investigators consider to be the definitive feature of a GPI.

These trifluoroacetic acid fragmentation and anion exchange procedures have been used to quantitate the Man to Man-6-P ratios in GPls biosynthetically labeled with [3H] Man. In addition, they are well suited to the detection and analysis of GPls radiolabeled exogenously by radiomethylation (Deeg et al., 1992, accompanying paper). Since this procedure places a stable radiolabel on amine components, putative GPI candidates can be fragmented for informative anion exchange and cation exchange chromatography. For example, radiomethylated trifluoroacetic acid fragments that chromatograph at the position of GlcN(Me)_2-Inos-P on anion exchange HPLC can subsequently be dephosphorylated with 50% HF and shown to chromatograph at the position of GlcN(Me)_2-Inos on cation exchange columns (Fig. 4B). This two-step sequence provides compelling evidence for the identification of a GlcN(Me)_2-Inos-P-containing GPI. The resolving power of cation exchange toward GPI components is high. In contrast to GlcN(Me)_2-Inos, for example, the initial HF-dephosphorylated product of α (predicted to be Man-Man-Man-GlcN(Me)_2-Inos) was not retained on the cation exchange column (Fig. 4C). The absence of negatively charged groups in this product was confirmed by its lack of retention on the Dionex anion exchange column (data not shown).

Future technical refinements may permit even more structural information to be obtained by Dionex anion exchange analysis of radiomethylated GPI fragments obtained under limited trifluoroacetic acid hydrolysis conditions. For example, fragments in peak d were noted under “Results” to be consistent with species that contain phosphorylated Man residues still linked to GlcN(Me)_2-Inos-P. The fact that peak d was eluted from the Dionex anion exchange column later than glycan α (compare Figs. 3B and 3E) indicated that the peak d fragments were more negatively charged than glycan α, an observation consistent with the absence of radiomethylated ethanolamine in peak d. Initial cleavage of two phosphodiester linkages between ethanolamine and phosphate in glycan α by trifluoroacetic acid would generate two mannose monophosphosphate moieties and increase the net negative charge by 2 in the residual glycan. Peak d elutes in a late, steep region of the acetate gradient that has not been optimized for maximal resolution, and it should be possible to resolve and identify these more highly phosphorylated radiomethylated trifluoroacetic acid fragments. Standards could then be established to deduce the number and location of ethanolamine phosphate groups in uncharacterized GPI species.

Acknowledgments—We thank Dr. Daniel Sevlever for helpful discussion during the course of this work and for quantitating the degradation of [3H]Man-6-P to [3H]Man by trifluoroacetic acid. We also are grateful to Tim Damon, John Foster, John Stein, and Jean Eastman for preparation of purified β^th ACHE.

**REFERENCES**

Baldwin, M. A., Stahl, N., Reinders, L. G., Gibson, B. W., Prusiner, S. B., and Burlingame, A. L. (1980) Anal. Biochem. 141, 174–182

Birringer, F., Rytwies, P. A., Shackleton, C., Brodebeck, U., and Stieger, S. (1990) J. Biol. Chem. 265, 18683–18687

Cross, G. A., M. M. (1990) Arne. Rev. Cell Biol. 6, 1–39

Deeg, M. A., Murray, N. R., and Rosenberry, T. L. (1992) J. Biol. Chem. 267, 18571–18588

Ferguson, M. A. J. (1992) Lipid Modification of Proteins: A Practical Approach (Turner, A. J., and Hooper, N., eds) pp. 191–230, IRL Press, Oxford

Ferguson, M. A. J., and Williams, A. F. (1986) Ann. Rev. Biochem. 57, 285–320

To avoid complications arising from background Man in the hydrolysis blanks (Footnote 5), this value was established by analysis of trifluoroacetic acid hydrolysates of [3H]Man-6-P isolated by Dionex PA-1 HPLC as outlined in Hirose et al. (1992).
Glycan Components in the Membrane Anchor of E<sup>na</sup> AChE

Ferguson, M. A. J., Low, M. G., and Cross, G. A. M. (1985) J. Biol. Chem. 260, 14547–14555

Ferguson, M. A. J., Homans, S. W., Dwek, R. A., and Rademacher, T. W. (1986) Science 230, 753–759

Gnagye, A. L., Forte, M., and Rosenberry, T. L. (1987) J. Biol. Chem. 262, 13280–13286

Haas, R., and Rosenberry, T. L. (1985) Anal. Biochem. 148, 154–162

Hass, R., Brandt, P. T., Knight, J., and Rosenberry, T. L. (1986) Biochemistry 25, 3096–3105

Hardy, M. R., Townsend, R. R., and Lee, Y. C. (1988) Anal. Biochem. 170, 54–62

Henner, D. J., Yang, M., Chen, W., Hellmiss, R., Rodriguez, H., and Low, M. G. (1988) Nucleic Acids Res. 16, 10383

Hirose, S., Prince, G. M., Sawleve, D., Ravi, L., Rosenberry, T. L., Ueda, E., and Medof, M. E. (1992) J. Biol. Chem. 267, 16968–16974

Homans, S. W., Ferguson, M. A. J., Dwek, R. A., Rademacher, T. W., Anand, R., and Williams, A. F. (1988) Nature 333, 269–272

Maltese, W. A. (1986) PASED J. 4, 5112–5128

McConville, M. J., and Bacic, A. (1989) J. Biol. Chem. 264, 757–766

Roberts, W. L., Myher, J. J., Kukas, A., Low, M. G., and Rosenberry, T. L. (1988a) J. Biol. Chem. 263, 15766–15770

Roberts, W. L., Santikarn, S., Reinhold, V. N., and Rosenberry, T. L. (1988b) J. Biol. Chem. 263, 15776–15784

Robert, W. L., Myher, K. J., Kukas, A., and Rosenberry, T. L. (1988c) Biochem. Biophys. Res. Commun. 150, 271–277

Rosenberry, T. L., and Scoggin, D. M. (1984) J. Biol. Chem. 259, 5563–5572

Rosenberry, T. L., Toutant, J.-P., Haas, R., and Roberts, W. L. (1989) Methods Cell Biol. 32, 241–255

Schneider, P., Ferguson, M. A. J., McConville, M. J., Mehlert, A., Homans, S. W., and Bordier, C. (1990) J. Biol. Chem. 265, 16955–16964

Selton, R. M., and Boss, J. E. (1987) J. Cell Biol. 104, 1449–1453

Smith, R., Braun, P. E., Ferguson, M. A. J., Low, M. G., and Sherman, W. R. (1987) Biochem. J. 248, 285–288

Turco, S. J., Orlandi, P. A., Jr., Homans, S. W., Ferguson, M. A. J., Dwek, R. A., and Rademacher, T. W. (1989) J. Biol. Chem. 264, 6711–6715

Walter, E. I., Roberts, W. L., Rosenberry, T. L., Ratnoff, W. D., and Medof, M. E. (1990) J. Immunol. 144, 1030–1038