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RESEARCH COMMUNICATION

Site of palmitoylation of a phospholipase C-resistant glycosyl-phosphatidylinositol membrane anchor

Michael A. J. FERGUSON
Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

The site of palmitoylation of the phosphatidylinositol moiety of the glycosyl-phosphatidylinositol membrane anchor of *Trypanosoma brucei* procyclic acidic repetitive protein was studied by using periodate oxidation. Analysis of the products by g.c.–m.s. allowed the assignment of 40 and 60% of the palmitate to the 2-position and the 3-position respectively of the *myo*-inositol ring.

INTRODUCTION

Glycosyl-phosphatidylinositol (GPI) membrane anchors are ubiquitous among the eukaryotes. Their primary function is to produce a stable association of cell-surface glycoproteins with the membrane, and other more specific functions have been proposed, reviewed most recently by Cross (1990) and Ferguson (1991). From the available structural data, reviewed by Ferguson (1991), it appears that all of these anchors are highly conserved with respect to the GPI core of:

ethanolamine-PO_4-6Manα1-2Manα1-6Manα1-4GlcNH_2-
α1-6*myo*-inositol-1-PO_4-lipid

where the ethanolamine is in amide linkage to the mature polypeptide *C*-terminal α-carboxy group. This conserved core structure may be variously substituted in a cell- and species-specific manner with extra ethanolamine phosphate and carbohydrate moieties. The lipid group can be either a glycerolipid or, in the case of yeast and *Dictyostelium*, a ceramide. In addition, some GPI anchors contain an additional fatty acid (palmitate) in a hydroxyester linkage to the inositol ring, which renders them resistant to the action of bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) and the GPI-specific phospholipase C (GPI-PLC) from *Trypanosoma brucei*. The basis of this phospholipase resistance was first described for human erythrocyte acetylcholinesterase (Roberts *et al.*, 1988), and subsequently for human erythrocyte decay accelerating factor (Walter *et al.*, 1990) and *T. brucei* procyclic acidic repetitive protein (PARP) (Field *et al.*, 1991). The precise location of the inositol-linked palmitate is unknown, but the fact that both PI-PLC and GPI-PLC cleavages result in the formation of an inositol 1,2-cyclic phosphate structure has led to the suggestion that occupation of the *myo*-inositol 2-position hydroxy group by palmitate would automatically preclude the action of these enzymes.

Here the position of the palmitate residue on the inositol ring has been analysed by periodate-oxidation studies on the GPI anchor of *T. brucei rhodesiense* PARP glycoprotein.

MATERIALS AND METHODS

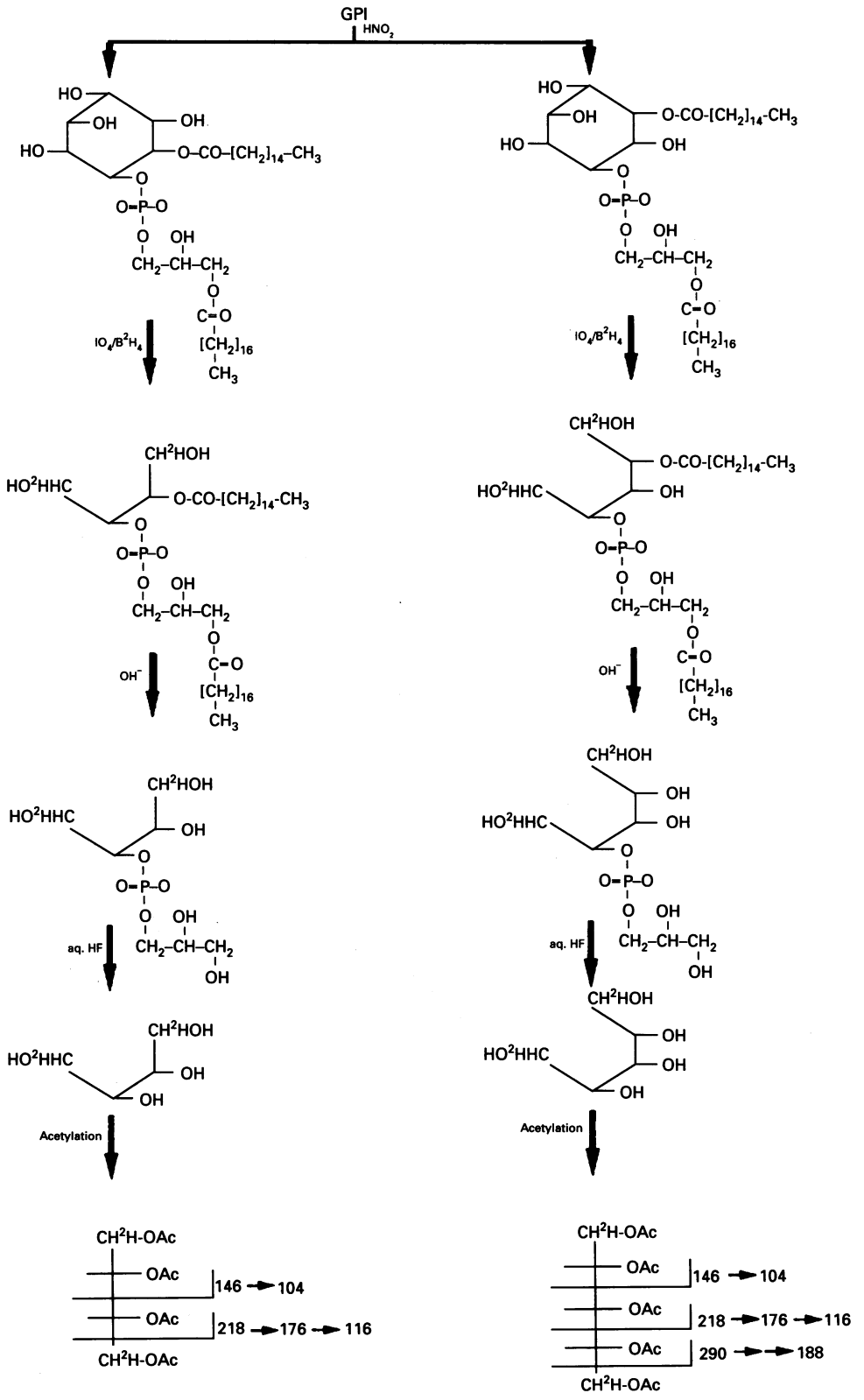
Preparation of the GPI-peptide of PARP

The PARP glycoprotein was purified from *T. brucei rhodesiense* procyclic cells which had washed three times in phosphate-buffered saline (0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.2) kindly given by Dr. Ian Maudlin, Tsetse Research Laboratory, University of Bristol, Bristol, U.K. The PARP glycoprotein was extracted from the wet cell pellet and purified by octyl-Sepharose (Pharmacia) chromatography (M. J. McConville, P. Murray & M. A. J. Ferguson, unpublished work; details available from M.A.J.F. on request) according to the method for the purification of *Leishmania* lipophosphoglycan (McConville *et al.*, 1990). The purified PARP was digested exhaustively with prolidase (Sigma), and the GPI-peptide was re-purified on octyl-Sepharose. The purified GPI-peptide was analysed for sugar, amino acid and inositol content and shown to contain *myo*-inositol and ethanolamine.

Deamination, periodate oxidation and product derivatization

A sample of the purified GPI-peptide fragment (10 nmol) was deaminated by the method of Güther *et al.* (1992), and the liberated palmitoylated phosphatidylinositol moiety was extracted into butan-1-ol. This material was dried, then oxidized by the sequential addition of 20 μl of propan-1-ol, 20 μl of 400 mM-sodium acetate buffer, pH 4.5, and (after sonication) 40 μl of 50 mM-sodium metaperiodate. The mixture was incubated for 48 or 120 h at 4 °C in the dark. The oxidation was terminated by the addition of 1 μl of ethylene glycol and incubation for 30 min at room temperature. The products were reduced by the addition of 30 μl of aq. 1 M-NH_3 followed by 60 μl of 1 M-NaB^2H_4 (1 h at room temperature). After acidification with 150 μl of 1 M-acetic acid and 15 μl of 6 M-HCl, the lipidic components were isolated by extracting twice with 100 μl of water-saturated butan-1-ol. After drying the butanol phase, the products were de-*O*-acylated with 100 μl of methanol, aq. 35% NH_3 (1:1, v/v) at 50 °C for 4 h. The products were dried, then dephosphorylated with 50 μl of aq. 48% (v/v) HF for 60 h at 0 °C. The digest was neutralized with 280 μl of satd. LiOH, and the LiF precipitate was removed by centrifugation. The LiF pellet was washed twice with 50 μl of water and the combined supernatants were desalted by passage through a column of 0.2 ml of AG50X12 resin (H^+ form) over 0.2 ml of AG3X4 resin (OH^- form) over 0.2 ml of QAE (quaternary aminoethyl)-Sephadex A25 (OH^- form) and elution with 3 ml of water. The eluate was dried and acetylated by the addition of 10 μl of 1-methylimidazole and 50 μl of acetic anhydride (20 min). The acetylation was terminated by the addition of 1 ml of water and, after 30 min, the acetylated products were recovered by extraction into 0.25 ml of chloroform.

Abbreviations used: GPI, glycosyl-phosphatidylinositol; GPI-PLC, GPI-specific phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C; PARP, procyclic acidic repetitive protein.



Scheme 1. Reaction scheme and products of the periodate-oxidation study

The major primary and secondary fragmentation ions for the final products of [1,4-^{2}H]erythritol tetra-acetate and [1,5-^{2}H]ribitol penta-acetate are indicated at the bottom of the Scheme.

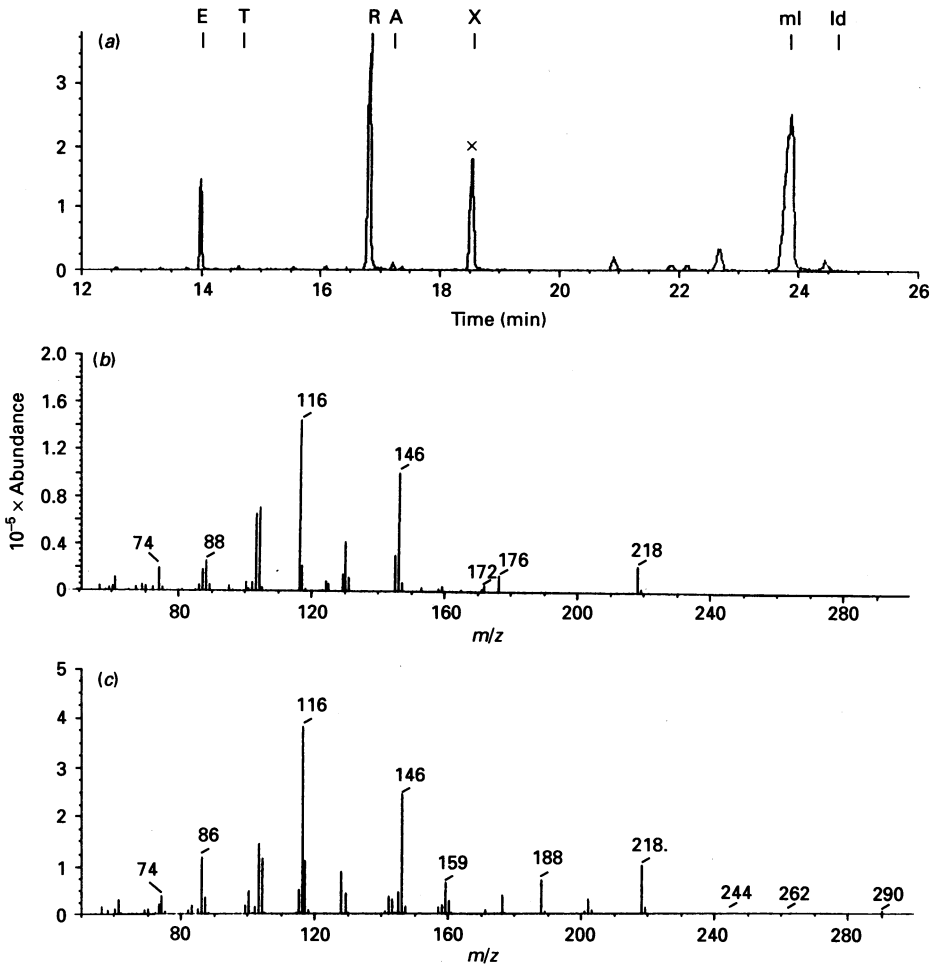


Fig. 1. G.c.–m.s. analysis of the periodate-oxidation products

(*a*) Extracted-ion chromatogram (*m/z* 116 plus *m/z* 210) of the alditol acetate products. The retention times of authentic standards are shown. E, T, R, A, X, mI and Id correspond to the acetates of erythritol, threitol, ribitol, arabinitol, xylitol, *myo*-inositol and iditol respectively. The peak marked ' × ' is a monodeuterated xylitol derivative. (*b*) Mass spectrum of peak E ([1,4-^{2}H]erythritol tetra-acetate). (*c*) Mass spectrum of peak R ([1,5-^{2}H]ribitol penta-acetate).

Table 1. Periodate oxidation products

A sample (10 μg) of phosphatidylinositol 4,5-bisphosphate (PIP_2) was oxidized under the conditions described in the text; the production of [1,6-^{2}H]iditol was monitored to estimate the efficiency of oxidation. The percentages of oxidation, and percentages of each product, were estimated from the molar quantities of inositol and alditols measured by g.c.–m.s. using total-ion-chromatogram integration and empirically determined molar-relative-response factors.

Substrate	Period of oxidation (h)	Oxidation (%)	Product (%)		
			Erythritol	Ribitol	Iditol
PIP_2	24	37	–	–	100
	48	55	–	–	100
	120	71	–	–	100
PARP-derived Palmitoyl-PI	48	46	40	60	–
	120	62	40	60	–

The chloroform phase was washed with 1 ml of water, dried under a stream of N_2 and redissolved in 20 μl of dichloromethane. Aliquots (1 μl) were analysed by g.c.–m.s.

G.c.–m.s.

The acetylated products were analysed by g.c.–m.s. using a Hewlett–Packard 5890-MSD instrument equipped with an SP2380 column (30 m × 0.25 mm; Supelco). Helium was used as carrier gas at 0.8 ml/min, and mass spectra were collected by using electron-impact and linear scanning (*m/z* 45–450). The temperature program was 120 (2 min) to 250 °C (20 min) at 10 °C/min. Extracted-ion chromatograms of ions at *m/z* 116 and

210 were used to identify [^{2}H]alditol and inositol acetates respectively. Alditol standards were obtained from Sigma.

RESULTS AND DISCUSSION

The reaction scheme and the results of the present study are summarized in Scheme 1. The extracted-ion chromatogram of the 48 h oxidation products (Fig. 1) shows the presence of some unoxidized *myo*-inositol hexa-acetate, together with [1,4-^{2}H]-erythritol tetra-acetate and [1,5-^{2}H]ribitol penta-acetate. The mass spectra of the latter two components are shown in Figs. 1(*b*) and 1(*c*). Dideuterated alditols can only arise from the oxidation and reduction of a cyclic polyol (in this case *myo*-inositol) and not from sugars, which can only produce monodeuterated alditols. The mass spectrum of the xylitol peak (results not shown) demonstrated that this alditol was monodeuterated, and therefore not the oxidation product of a cyclic polyol. The extent of *myo*-inositol oxidation, and the relative molar ratios of the products after 48 and 120 h of periodate treatment, are given in Table 1. Assuming that the *myo*-inositol ring is substituted at the 1-position by the glycerophosphate moiety, the stereochemistry of the *myo*-inositol ring dictates that [1,4-^{2}H]erythritol and [1,5-^{2}H]ribitol can be generated only via periodate oxidation and NaB^2H_4 reduction of *myo*-inositol further substituted at the 2- and 3-positions respectively. The possibility that ribitol is a partial oxidation product can be ruled out by the data in Table 1, which shows that the ratio of ribitol to erythritol is independent of the total extent of *myo*-inositol oxidation.

These data unambiguously define the site of palmitoylation as the 2-position (40%) and the 3-position (60%) of the *myo*-inositol ring of the isolated phosphatidylinositol moiety. This can be explained in one of two ways: (i) the GPI anchor was originally palmitoylated by the cell at the *myo*-inositol 2-position *and* the 3-position, or (ii) the GPI anchor was originally palmitoylated by the cell exclusively at the *myo*-inositol 2-position *or* the 3-position. The second model could be explained, in terms of the experimental data, by acyl migration of the palmitate ester between the *myo*-inositol 2-position and the 3-position. The acidic conditions of the deamination reaction and the periodate oxidation could favour such acyl migration between these *vicinal cis*-hydroxy groups; further acyl migration around the ring would not be expected, since the 3-position and 4-position hydroxy groups are *trans* to each other.

Although a completely unambiguous answer to the problem is not possible using this approach, it may be concluded that palmitoylatoin of the *myo*-inositol ring occurs at only the 2-position and/or the 3-position.

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