The nature and extent of carbohydrate modification in intraerythrocytic stage Plasmodium falciparum proteins have been controversial. This study describes the characterization of the carbohydrates in intraerythrocytic P. falciparum proteins and provides an overall picture of the nature of carbohydrate modification in the parasite proteins. P. falciparum strains were metabolically labeled with radioactive sugar precursors and ethanolamine at different developmental stages. The individual parasite proteins separated on SDS-polyacrylamide gels and whole parasite cell lysates were analyzed for the carbohydrate moieties. The results established the following: 1) glycosylphosphatidylinositol (GPI) anchors represent the major carbohydrate modification in the intraerythrocytic stage P. falciparum proteins; 2) in contrast to previous reports, O-linked carbohydrates are either absent or present only at very low levels in the parasite; and 3) P. falciparum contains low levels of N-glycosylation capability. The amount of N-linked carbohydrates in whole parasite proteins is ~6% compared with the GPI anchors attached to proteins based on radioactive GlcN incorporated into the proteins.

The glycans of multiple parasite protein GPI anchors are all similar, consisting of protein-ethanolamine-phosphate-(Manα1-2)6Manα1-2Manα1-6Manα1-4GlcN. The fourth Man residues distal to GlcN of the GPI anchor glycans contain unidentified substituents that are susceptible to conditions of nitrous acid deamination. This unusual structural feature may contribute to the reported pathogenic properties of the P. falciparum GPI anchors.

Malaria, a life-threatening disease caused by parasitic protzoa of the genus Plasmodium, is a major health problem throughout the tropical and subtropical regions of the world. Among the four species that infect humans, Plasmodium falciparum is the most virulent. New approaches such as vaccine development and novel therapeutic agents are urgently needed due to the emergence of parasite strains resistant to chloroquine and other commonly used drugs (1).

Vaccines based on antigens of the blood stage parasite are under intensive study. A major focus has been on synthetic peptides or recombinant proteins of cell surface antigens (2–6). However, this approach has not been highly effective, although immunization with native cell surface proteins purified from the erythrocytic stage parasite is known to confer significant protective immunity (7–10). It is plausible that post-translational modifications play an important role in antigenicity. Accordingly, a basic understanding of such modifications may assist in the development of effective vaccines.

Glycosylation is an often extensive post-translational modification of eukaryotic proteins. Carbohydrate moieties of glycoproteins perform a variety of functions including modulation of immunological properties, receptor-ligand interactions, sorting and localization of proteins, cell adhesion, and cell-cell communication (11). In addition, they contribute to protein conformation and, thus, to proteolytic processing (11). Finally, the carbohydrate moieties can be highly antigenic and may contribute to disease pathology.

Several proteins of erythrocytic stage P. falciparum are known to contain carbohydrates (12–23). The deduced amino acid sequences of the parasite proteins have potential N-glycosylation sites (7, 24–26). However, Dieckmann-Schuppert et al. (27) have reported that P. falciparum has no N-glycosylation capability based on their finding that dolichol phosphate-linked oligosaccharides, the obligatory biosynthetic intermediates, and dolichol phosphate-oligosaccharyltransferase activity were not detectable in the parasite. However, while this work was in review, Kimura et al. (28) reported the existence of N-linked glycans in P. falciparum.

Two laboratories have previously reported that O-glycosylation is the major carbohydrate modification in proteins of the intraerythrocytic asexual stage of P. falciparum (21, 23) and that the O-linked carbohydrates are mainly single residues of GlcNAc with the remainder being oligosaccharides, some containing GlcNAc at the reducing end. While one group reported that the parasite proteins also contain O-linked GalNAc (19, 21), another group showed the absence of this sugar moiety and the lack of its biosynthesis (23). Kimura et al. reported the presence of O-glycanase-releasable oligosaccharides as the major carbohydrates in the proteins of late trophozoite and schizont stage P. falciparum (28).

Previous studies reported the presence of glycosylphosphatidylinositol (GPI)1 in several proteins of erythrocytic stage P. falciparum including merozoite surface protein 1 (MSP-1),

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1 The abbreviations used are: GPI, glycosylphosphatidylinositol; GlcNAcol, N-acetylglucosaminol; AHM, 2,5-anhydromannitol; GU, glucose units; GalT, N-acetylgalactosaminyl β1,4-galactosyltransferase; GlcNAcT, N-acetylgalactosaminytransferase; MSP, merozoite surface protein; BCA, bicinchoninic acid; PMSF, phenylmethylsulfonyl fluoride; TPCK, t-1-p-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, N,N',p-tosyl-l-lysine chloromethyl ketone; HF, hydrofluoric acid; HPAEC, high pH anion exchange chromatography; HPTLC, high performance
merozoite surface protein 2 (MSP-2), 72-kDa heat shock protein (HSP-72), 102-kDa transferrin receptor, and 75-kDa serine protease (29–32). Recently, Gerard et al. (33) studied the GPI lipids (not anchored to proteins) of P. falciparum. Two putative GPI anchor precursors, ethanolamine-phosphate-(Manα1–2Manα1–2Manα1–4GlcN-PI and ethanolamine-phosphate-6Manα1–2Manα1–6Manα1–4GlcN-PI, were identified (33). While the current study was in progress, Gerard et al. (34) reported the structures of the GPI anchors of P. falciparum MSP-1 and MSP-2. Both were shown to consist of ethanolamine-phosphate-(Manα1–2Manα1–6Manα1–4GlcN-1-phosphatidylinositol, bearing a myristic acid substitution on the inositol ring and predominantly palmitic acid in the diacylglycerol moiety (34).

In this study, using several P. falciparum strains, we clearly demonstrate that O-linked carbohydrates are either absent or present only at very low levels in intraerythrocytic P. falciparum proteins. In agreement with Kimura et al. (28), we show that P. falciparum apparently contains N-linked carbohydrates. However, in contrast to the former study (23), our results establish that N-linked oligosaccharides are minor constituents, and that GPI anchors represent the major carbohydrate modification in intraerythrocytic P. falciparum proteins. Furthermore, we establish that GPI anchors of the parasite proteins have Manα1–4GlcN cores with substituents that are susceptible to the conditions of nitrous acid deamination on the terminal Man residues. These unusual structures may contribute to the reported cellular and immunological functions of P. falciparum GPI anchors (35, 36).

EXPERIMENTAL PROCEDURES

Materials—Aspergillus saitoi α-mannosidase (400 milliliters/mg), jack bean α-mannosidase (50 units/mg), bovine milkGalT, and a mixture of Gc oligomers were from Oxford Glycosystems (Rosedale, NY); PMSF and (4-amidinophenyl)-methylsulfonyl fluoride hydrochloride were from Boehringer Mannheim; N-glycanase was from New England Biolabs (Beverly, MA); [1-3H]ethanolamine hydrochloride (10–30 Ci/mmol), [6-3H]Gal (40–60 Ci/mmol), [6-3H]Man (20–30 Ci/mmol), UDP-[6-3H]Gal (40–60 Ci/mmol), GDP[3H]Gal (40–60 Ci/mmol), GDP[N3H]N-acetylglucosamine (40–60/Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO); rainbow protein molecular weight markers, rainbow 14C-methylated protein molecular weight markers, and AmplifyTM fluorographic solution were from Amersham Corp.; Bio-Gel P-4 (fine and extra fine), Bio-Gel P-100 (fine and extra fine), Bio-Gel P-200, and Bio-Gel P-400, and incubated with Pronase (5 mg; 1-mg aliquots were added at intervals of 8–12 h) at 4°C for 24 h (45). The reaction mixture was cooled in an ice bath and neutralized with 2 M HOAc, and the gel was washed with water (3 ml). The combined reaction solution was centrifuged at 10,000 × g for 10 min, and then with MeOH, water, and ethanolamine [3H]GlcN-labeled carbohydrates were recovered from the 200–215-, 82-, and 66-kDa protein bands in the SDS-polyacrylamide gels, visualized by salicylic acid-enhanced fluorography (44), were excised, and the filter paper backing was scraped off. The procedure outlined here is for a single protein band. For large scale isolation, corresponding protein bands from different lanes were comibined, and reagents were appropriately scaled up. The gels were cut into 1-mmlong pieces and suspended in water (1 ml). The excess water was removed, and the gel was washed with water (3 × 1 ml) to remove the radioactivity enhancer (44). In some instances, water-swollen gel pieces were washed with MeOH (3 × 1 ml), which also removes the enhancer effectively. The water-swollen gel pieces were treated with an equal volume of 100 mM NaOH, 1 mM NaBH4, diluted to 2 ml with 50 mM NaOH, 0.5 mM NaBH4, and incubated at 44°C for 24 h (45). The reaction mixture was cooled in an ice bath and neutralized with 2 mM HOAc, and the clear solution was removed. The gel pieces were washed with water (5 × 2 ml) and then with MeOH (3 × 2 ml). The combined reaction solution and washes were centrifuged at 10,000 × g to remove insoluble particles. The supernatant was then dried in a rotary evaporator at 35°C. Boric acid was removed by evaporation with 0.1% HOAc in MeOH (3 × 4 ml). The residue was dissolved in water and chromatographed on Bio-Gel P-4 (see below). Typically, 20,000–30,000 cpm of [3H]GlcN-labeled carbohydrates were recovered from the 200–215-, 82-, and 75-kDa protein bands.

Isolation of Intact GPI Anchor of Parasite Proteins Separated on Polyacrylamide Gels—The [3H]labeled parasite protein bands in the SDS-polyacrylamide gels, visualized by salicylic acid-enhanced fluorography (44), were excised, and the filter paper backing was scraped off. The procedure outlined here is for a single protein band. For large scale isolation, corresponding protein bands from different lanes were combined, and reagents were appropriately scaled up. The gels were cut into 1-mmlong pieces and suspended in water (1 ml). The excess water was removed, and the gel was washed with water (3 × 1 ml) to remove the radioactivity enhancer (44). In some instances, water-swollen gel pieces were washed with MeOH (3 × 1 ml), which also removes the enhancer effectively. The water-swollen gel pieces were treated with an equal volume of 100 mM NaOH, 1 mM NaBH4, diluted to 2 ml with 50 mM NaOH, 0.5 mM NaBH4, and incubated at 44°C for 24 h (45). The reaction mixture was cooled in an ice bath and neutralized with 2 mM HOAc, and the clear solution was removed. The gel pieces were washed with water (5 × 2 ml) and then with MeOH (3 × 2 ml). The combined reaction solution and washes were centrifuged at 10,000 × g to remove insoluble particles. The supernatant was then dried in a rotary evaporator at 35°C. Boric acid was removed by evaporation with 0.1% HOAc in MeOH (3 × 4 ml). The residue was dissolved in water and chromatographed on Bio-Gel P-4 (see below). Typically, 20,000–30,000 cpn of [3H]GlcN-labeled carbohydrates were recovered from the 200–215-, 82-, and 75-kDa protein bands.

Isolation of Intact GPI Anchor of Parasite Proteins Separated on Polyacrylamide Gels—The protein bands in the SDS-polyacrylamide gel slices were cut into small pieces, washed with water and MeOH, and then dried in a Speed-Vac. The samples were suspended (0.5 ml) and homogenized to a fine paste using a glass minihomogenizer. The pastes (from two bands of the same protein) were suspended in 4 ml of 100 mM Tris-HCl, 1 mM CaCl2, pH 8.0, containing 0.05% SDS and 0.5% Nonidet P-40, and incubated with Pronase (5 mg; 1-ml aliquots were added at intervals of 8–12 h) at 55°C for 60 h. The enzyme digests were centrifuged, and the gels were washed with water (3 × 2 ml) and then with

thin layer chromatography; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
**Glycosylation of P. falciparum Proteins**

MeOH (2 × 2 ml). The combined supernatants and washings were dried in a rotary evaporator at 30 °C. The residues were dissolved in water (0.5 ml) and then each chromatographed on Bio-Gel P-4 (1 × 90 cm) in 100 mM pyridine, 100 mM NaOAc, pH 5.2, or extracted with water-saturated 1-butanol (4 × 0.5 ml) (46–49). The 1-butanol layers were extracted with a Percoll cushion as described above. The three times with ChCl/MeOH, water (10:10:3, v/v/v) to remove glycoproteins containing free GPI-anchors. The pellet was dissolved in 25 mM Tris-HCl, pH 7.5, containing 1% SDS, 10 mM benzamidine, 5 mM EDTA, 1 mM PMFS, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 2 μg/ml antipain, and 1 μg/ml chymostatin, and dialyzed (M5, cut-off of 6,000–8,000) extensively for 48 h above the buffer followed by dialysis for 1 h against distilled water containing protease inhibitors. The lysates were lyophilized, washed with 90% MeOH, and then subjected to 1) alkaline β-elimination, 2) digestion with Pronase, or 3) digestion with N-glycanase.

**Preparation of Delipidated P. falciparum Cell Lysates—**The parasite cultures at early and late trophozoite stages were labeled with [3H]GlcN and were separately dried in a Speed-Vac. The residue from the 1-butanol extracted with water-saturated 1-butanol (3×) were similarly chromatographed. Fractions containing radiolabeled carbohydrates were pooled, lyophilized, and deionized with AG 50W-X12 (H+) and AG 4-X4 (base).

**Isolation of GPI Glycan Core—**The [3H]GlcN-labeled GPI anchors obtained by the Pronase digestion and extraction with 1-butanol were treated, before and after N-acetylation, with 25 mM sodium periodate in 50 mM NaOAc, pH 5.5 (200 μl) for 16 h at room temperature in the dark. Excess periodate was destroyed by the addition of glycerol (10 μl), and the products were chromatographed on a column of Bio-Gel P-4 in 100 mM pyridine, 100 mM NaOAc, pH 5.2.

**Deamination with HNO2—**The [3H]GlcN-labeled GPI anchors obtained by the Pronase digestion and extraction with 1-butanol were treated, before and after N-acetylation, with 25 mM sodium periodate in 50 mM NaOAc, pH 5.5 (200 μl) for 16 h at room temperature in the dark. Excess periodate was destroyed by the addition of glycerol (10 μl), and the products were chromatographed on a column of Bio-Gel P-4 in 100 mM pyridine, 100 mM NaOAc, pH 5.2.

**Gel Filtration Chromatography—**Gel filtration of radioactive components was carried out in an acid water solution containing 100 mM Tris-HCl, 1% SDS, 10 mM benzamidine, 5 mM EDTA, 1 mM PMFS, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 2 μg/ml antipain, and 1 μg/ml chymostatin, was dialyzed (M5, cut-off of 6,000–8,000) extensively for 48 h above the buffer followed by dialysis for 1 h against distilled water containing protease inhibitors. The lysates were lyophilized, washed with 90% MeOH, and then subjected to 1) alkaline β-elimination, 2) digestion with Pronase, or 3) digestion with N-glycanase.

**Alkaline-Sodium Borohydride Treatment of P. falciparum Cell Lysate—**The parasite cell lysate (~120,000 cpm) was dissolved in 50 mM NaOH, 0.5 mM NaBH4 (2 μl), incubated at 44 °C for 22 h, and then neutralized with 2 M HOAc to 0.5 ml containing 0.5% SDS, 1% β-mercaptoethanol and then heated in a boiling water bath for 10 min and cooled. Then 0.5 mM sodium phosphate, pH 7.5 (35 μl), and 10% Nonidet P-40 (35 μl) were added. The suspension was sonicated in an ultrasonic bath for 10 min to obtain a clear solution, and then N-glycanase (400 IU3 milliliters) was added and incubated at 37 °C for 36 h. Four volumes of MeOH were added, and the solution was chilled on dry ice for 10 min and then centrifuged. The supernatant was dried in a Speed-Vac, and the residue was dissolved in water and extracted with water-saturated 1-butanol to remove the detergents. The aqueous phase was chromatographed on Bio-Gel P-4 (1 × 90 cm) in 0.1 M pyridine, 0.1 M HAc, pH 5.2. Fractions containing radioactivity were pooled and lyophilized.

**Digestion of P. falciparum Cell Lysate with N-Glycanase—**The [3H]GlcN-labeled parasite lysate (~110,000 cpm) was suspended in 300 μl of 0.5% SDS, 1% β-mercaptoethanol and then heated in a boiling water bath for 10 min and cooled. Then 0.5 mM sodium phosphate, pH 7.5 (35 μl), and 10% Nonidet P-40 (35 μl) were added. The suspension was sonicated in an ultrasonic bath for 10 min to obtain a clear solution, and then N-glycanase (400 IU3 milliliters) was added and incubated at 37 °C for 36 h. Four volumes of MeOH were added, and the solution was chilled on dry ice for 10 min and then centrifuged. The supernatant was dried in a Speed-Vac, and the residue was dissolved in water and extracted with water-saturated 1-butanol to remove the detergents. The aqueous phase was chromatographed on Bio-Gel P-4 (1 × 90 cm) in 0.1 M pyridine, 0.1 M HAc, pH 5.2. The radioactive fractions were pooled and lyophilized, and their carbohydrate compositions were determined before and after reduction with NaBH4.

**Gel Filtration of Radioactive Components—**The [3H]GlcN-labeled parasite lysate (~110,000 cpm) was suspended in 100 mM Tris-HCl, 1 mM CaCl2, pH 8.0 (0.5 ml) and incubated with Pronase (4 × 1 mg, added at 10–12 h intervals) at 37 °C for 48 h. The solution was extracted with water-saturated 1-butanol (3 × 0.5 ml), and the two phases were separately dried in a Speed-Vac. The residue from the 1-butanol phase was analyzed for radioiodinated carbohydrates by HPAEC before and after treatment with PNGase F. The aqueous phase was chromatographed on a Bio-Gel P-4 (1 × 90 cm) in 0.1 M pyridine, 0.1 M HAc, pH 5.2. The eluted radioactive components were analyzed for carbohydrates.

**Analysis of P. falciparum Proteins for Terminal GlcNAc Residues by Galactosylation—**Erythrocytes harboring trophozoite and schizont stage parasites were labeled by centrifugation on a Percoll cushion and lysed with 0.015% saponin(18). The released parasites were washed with PBS and then lysed with 25 mM HEPES, pH 7.3, containing 0.05% SDS, 1% Nonidet P-40, 0.1 mM PMFS, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml chymostatin, 2 μg/ml antipain, and 5 mM benzamidine. To the parasite cell lysate containing 100 μg of protein, 20 μl of 100 mM HEPES, pH 7.3, containing 100 mM Gal, and 50 mM MnCl2 100 milliliters of GaT, 2 μl of 100 × protease inhibitor stock solutions. The solution was diluted with water (180 μl), 5 μCi of UDP-[3H]Gal in water (20 μl) containing 25 mM AMP was added, and the solution was incubated at 37 °C for 2 h (50). The reaction was stopped by the addition of 100 mM EDTA, 10% SDS (20 μl). Aliquots of the reaction mixture containing 30–40 μg of parasite proteins were analyzed by SDS-PAGE fluorography using a 7–20% SDS-polyacrylamide gel.

**Cruze erythrocyte ghosts were prepared by the lysis of human red cells with 1:20 diluted phosphate-buffered saline for 30 min, centrifuged, and washed with the same buffer. The pellet was suspended in 25 mM HEPES, 1% Nonidet P-40, pH 7.3. The detergent-solubilized human erythrocyte ghosts and ovalbumin were galactosylated in parallel as positive controls and analyzed by SDS-PAGE fluorography.**
200–215- and 82-kDa proteins (20,000–30,000 cpn) isolated as above were dried in reative vials and peracetylated with 40 μl of pyridine, acetic anhydride (1:1, v/v) at room temperature for 18 h (47–49). The solutions were dried in a Speed-Vac and then treated with 30 μl of acetic anhydride, HOAc, concentrated sulfuric acid (10:10:1, v/v/v) for 8 h at 37 °C (47–49). To each reaction mixture, 10 μl of pyridine and 500 μl of water were added and then extracted with CHCl3 (250 μl). The organic layers were washed with water, and treated with 30% ammonia (200 μl), MeOH (1:1, v/v) at 37 °C for 24 h. The solutions were dried in a Speed-Vac, and the residues were dissolved in water and then chromatographed on Bio-Gel P-4 in 100 mM pyridine, 100 mM HOAc, pH 5.2.

Partial Hydrolysis—The [3H]GlcN-labeled GPI anchors (60,000–100,000 cpn) isolated by NaOH/NaBH4 treatment of 200–215- and 82-kDa protein bands were deaminated with HNO2 and reduced with NaBH4. The samples were dissolved in 0.1 M trifluoroacetic acid (400 μl), heated at 100 °C for 4 h (46, 49), and then dried in a Speed-Vac. About half of the partial hydrolysates were treated with 25 μl (30 units/ml) of jack bean α-mannosidase, deionized with AG 50W-X16 (H+), and then dried. The enzyme-treated and untreated partial hydrolysates were dephosphorylated with 50% aqueous HF and then deionized with 100 mM NaOH at a flow rate of 0.8 ml/min, for hexosamines on CarboPac PA1, and 3) 100 mM NaOH for 10 min and then maintenance of 40% of 100 mM NaOH, 250 mM NaOAc at sample injection and then a linear gradient to 40% of 100 mM NaOH, 250 mM NaOAc at 35 min and then maintenance of 40% of 100 mM NaOH, 250 mM NaOAc for 10 min. Fractions (0.4 ml) were collected, and the radioactivity was measured. Glc oligomers were added to samples as internal standards, and were monitored by pulsed amperometric detection.

The [3H]GlcN-labeled parasite GPI anchors were separated and identified by comparison of elution time with those of Glc oligomers and standard AHM, Man-AHM, Man2-AHM, Man3-AHM, and Man4-AHM.

TLC Analysis of GPI Anchors—The glycan cores of the parasite GPI anchors, their partial hydrolysates, and the products of exoglycosidase digestion and partial acetolysis were also analyzed by HPTLC (46, 49). The plates were developed with 1-propanol, acetone, water (9:6:4, v/v/v), dried, sprayed with EN3HANCE, and then exposed to x-ray film at −70°C. The parasite GPI glycans were identified by comparison of retention times with those of Man3-AHM standard and an AHM to Man4-AHM ladder.

Treatment with Jack Bean α-Mannosidase—GPI glycans were treated with 25–50 μl of jack bean α-mannosidase (30 units/ml) in 100 mM NaOAc, pH 5.0, containing 2 mM Zn2+ at room temperature for 2 h and then at 37 °C for 22 h (46, 47, 49). The solutions were heated in a boiling water bath for 5 min and desalted either with AG 50W-X16 (H+), resin or by gel filtration on Bio-Gel P-4 columns (1 × 90 cm) in 100 mM pyridine, 100 mM NaOAc, pH 5.2.

Treatment with A. saitoi α-Mannosidase—The GPI glycans were incubated with 20 μl of A. saitoi α-mannosidase (1 million unit/ml) in 100 mM NaOAc, pH 5.0, at 37 °C for 22 h (46, 47, 49). The solutions were heated in a boiling water bath for 5 min and desalted with AG 50W-X16 (H+) and AG 4X4 (base) resins.

**RESULTS**

Metabolic Labeling and Analysis of Glycosylated *P. falciparum* Proteins—Intraerythrocytic stage *P. falciparum* was metabolically labeled with [3H]GlcN, [3H]Man, [3H]Gal, or [3H]Fuc in medium containing 5 mM D-Glc and 10% human serum. SDS-PAGE analysis of the parasitized erythrocyte cell lysates and fluorographic identification of the protein bands demonstrate that [3H]GlcN was incorporated into more than 15 proteins (size range, 14–260 kDa) (Fig. 1). [3H]Man gave a similar labeling pattern, but the incorporation of radioactivity was only about 5–10% of that observed with [3H]GlcN (data not shown).
[\textsuperscript{3}H]Gal and [\textsuperscript{3}H]Fuc were not significantly incorporated into the parasite proteins (data not shown). Since the incorporation of [\textsuperscript{3}H]Man to the parasite proteins was low, subsequent metabolic labeling was routinely performed with [\textsuperscript{3}H]GlcN.

Metabolic labeling with [\textsuperscript{3}H]GlcN was performed at different developmental stages of the parasite (rings undergoing transformation to schizonts) (Fig. 1). Among the radiolabeled parasite proteins (Fig. 1A), 200–215-, 82-, and 75-kDa proteins were predominant. Two proteins (36 and 53 kDa) were labeled relatively intensely by the ring stage parasites, and their intensity drastically decreased in the trophozoites and schizonts. The incorporation of [\textsuperscript{3}H]GlcN by the schizonts was significantly lower (Fig. 1A, lanes 6 and 7) compared with the rings and trophozoites (Fig. 1A, lanes 1–5). At least four radiolabeled proteins (38, 43, 46, and 56 kDa, some not well resolved on the gel) appear to be synthesized only at the trophozoite and schizont stages (Fig. 1A, compare lanes 2–7 with lane 1). A 260-kDa protein band was labeled to a significant level only by the ring stage parasites (Fig. 1A, lane 1). Several minor proteins with molecular weights ranging from 130,000 to 200,000 were also labeled with [\textsuperscript{3}H]GlcN. In contrast to a previous report (23), noninfected red cells did not incorporate radiolabeled precursors (Fig. 1B, lane 4). >99% of the incorporated radioactivity was parasite-dependent. Previously, Udienza and Van Dyke (18) and others (28) reported that noninfected erythrocytes do not incorporate [\textsuperscript{3}H]GlcN into proteins.

To determine whether the carbohydrate modification of \textit{P. falciparum} proteins differs between strains, three other parasite strains, W2, D6, and NF54, were also metabolically labeled with [\textsuperscript{3}H]GlcN. SDS-PAGE of the cell lysates and fluorography revealed similar protein labeling patterns to that observed with the FCR-3 strain, with the exception of the altered mobility of the 75- and 82-kDa proteins (Fig. 1, compare panel A, lane 1, with panel B). In the W2 strain, the mobility of these two proteins was slower than in the FCR-3 strain, and they electrophoresed as two distinct, widely separated bands (Fig. 1B, lane 2). In the D6 strain, the mobility of the 75- and 82-kDa proteins (Fig. 1B, lane 1) was comparable with the respective proteins of the FCR-3 strain. These proteins appear as a single band in the NF54 strain (Fig. 1B, lane 3). The difference in mobility of 75- and 82-kDa proteins from different parasite strains is not due to altered glycosylation, since they have similar carbohydrate moieties (see below).

\textbf{Analysis of SDS-PAGE-separated \textit{P. falciparum} Proteins for Carbohydrate Moieties—}After SDS-PAGE of the parasite lysates and fluorography, the individual [\textsuperscript{3}H]GlcN-labeled protein gel bands were separately excised and washed with water to remove the radioactive enhancer. More than 98% of the radiolabeled proteins remained in the gel slices. Pronase digestion (200–215-, 82-, and 75-kDa proteins from the FCR-3 strain) or alkaline borohydride treatment (all radiolabeled proteins from four parasite strains) released more than 95% of the radiolabeled carbohydrate moieties from the gel slices. These were isolated by gel filtration using Bio-Gel P-4 (Figs. 2 and 3). Approximately 95% of the Pronase-released carbohydrate eluted in the void volume along with the detergents from the reaction mixture, and the remainder was eluted at a 1200–1600-Da range (Fig. 2). However, after treatment with mild alkali or HNO\textsubscript{2}, all of the radioactivity was eluted at a 1200–1600-Da range (Fig. 2).

The radiolabeled carbohydrates released by NaOH/NaBH\textsubscript{4} treatment of 200–215-, 43–46-, and 14-kDa protein bands eluted on Bio-Gel P-4 as single symmetrical peaks corresponding to a molecular weight of 1200–1600 (Fig. 3). In contrast, the radiolabeled carbohydrates from the 36–38-, 53–56-, 75-, and 82-kDa protein bands (FCR-3 strain) eluted as broad (in some cases heterogeneous) peaks at a 1200–2400-Da range (Fig. 3C). Upon digestion with Pronase, however, all of the samples were eluted as symmetrical peaks at a molecular weight range similar to that from the 200–215-kDa protein (Fig. 3D), suggesting that the observed higher sizes were due to peptide moieties associated with the carbohydrates. Therefore, the release of radiolabeled carbohydrates from parasite proteins by NaOH/NaBH\textsubscript{4} is not due to classical β-elimination of Ser/Thr-linked O-glycosidic bonds but apparently to alkaline degradation of protein moieties. The [\textsuperscript{3}H]GlcN-labeled peptide-carbohydrate moieties obtained from various proteins of the D6, W2, and NF54 strains were also analyzed (data not shown). In all cases, the results were similar to those from the FCR-3 strain. In no case did significant radioactivity elute at the hydrodynamic volume corresponding to hexosamines or hexosaminoligos.


**FIG. 3.** Bio-Gel P-4 chromatography of carbohydrate moieties of *P. falciparum* proteins released by NaOH/NaBH₄. [³H]GlcN-labeled protein bands from SDS-polyacrylamide gels were treated with 100 mM NaOH, 0.5 mM NaBH₄, as described under "Experimental Procedures." Radioactive components released (50,000–60,000 cpm) were chromatographed on Bio-Gel P-4 columns (1 × 90 cm) in 100 mM pyridine, 100 mM HOAc, pH 5.2. Fractions (1.1 ml) were collected, and 60-μl aliquots were measured for radioactivity in a liquid scintillation counter. Shown are the chromatograms of the carbohydrate moieties released from the 200–215-kDa (A) and 82-kDa (B) proteins before (closed circles) and after (open circles) treatment with Pronase. C, chromatographic profiles of carbohydrate moieties released from 75-kDa (open squares), 53–56-kDa (open circles), 43–46-kDa (closed circles), and 14-kDa (closed squares) proteins. D, chromatographic profiles of carbohydrates in C after digestion with Pronase. Carbohydrate from 75-kDa (open squares), 53–56-kDa (open circles), 43–46-kDa (closed circles), and 14-kDa (closed squares) proteins. Elution positions of blue dextran (V₀) and water-saturated 1-butanol. After deamination, ~90% of the radioactivity remained in the aqueous phase, and the remainder partitioned into the 1-butanol layer. A retreatment of the radioactivity in the organic phase with HNO₂ partitioned almost all of the carbohydrate moieties into the aqueous phase. GlcN was barely detectable after deamination of the carbohydrate moieties of parasite proteins. In all parasite proteins analyzed, GlcN was converted into 2,5-AHM (identified as 2,5-anhydromannitol; see below) after treatment with HNO₂.

These results indicate that the carbohydrate moieties of parasite proteins contain predominantly GlcN. This finding was confirmed for all *P. falciparum* strains used in this study.

The presence of GlcN in the carbohydrates of parasite proteins was further supported by periodate oxidation studies. Dionex-HPAEC analysis of the acid hydrolysates of periodate-oxidized products revealed that the GlcN residues were almost quantitatively destroyed. However, upon N-acetylation, the GlcN residues were completely resistant to periodate oxidation.

The predominance of GlcN with little or no N-acetylation of most of the predominantly radiolabeled carbohydrate moieties of parasite proteins (from FCR-3, D6, W2, and NF54 strains) suggests that GPI anchors are the major carbohydrate moieties of the parasite proteins. To verify whether this is the case, the parasites were metabolically labeled with [³H]ethanolamine. SDS-PAGE of the cell lysates and fluorography gave a labeling pattern of proteins similar to that observed with [³H]GlcN labeling (data not shown). The Bio-Gel P-4 chromatography of the [³H]ethanolamine-labeled components released from the protein gel bands, either by Pronase or alkaline borohydride, gave elution patterns similar to the carbohydrate moieties obtained from [³H]GlcN-labeled parasite proteins (data not shown).

**TABLE I**

| Protein band | Relative proportions of the [³H]GlcN-labeled carbohydrate moieties | Hexosamine detected | Nature of the [³H]GlcN-labeled carbohydrate moiety detected |
|--------------|---------------------------------------------------------------|--------------------|---------------------------------------------------|
| 200–215 kDa  | 28.5 GlcN                                                 |                   | GP⁺ GlcN                                         |
| 82 kDa       | 33.8 GlcN                                                 |                   | GP⁺ GlcN                                         |
| 75 kDa       | 26.6 GlcN                                                 |                   | GP⁺ GlcN                                         |
| 53–56 kDa    | 3.1 GlcN                                                  |                   | GP⁺ GlcN                                         |
| 43–48 kDa    | 2.2 GlcN                                                  |                   | GP⁺ GlcN                                         |
| 36–38 kDa    | 3.3 GlcN                                                  |                   | GP⁺ GlcN                                         |
| 14 kDa       | 2.5 GlcN                                                  |                   | GP⁺ GlcN                                         |

*Approximate relative proportions of the radioactive carbohydrate moieties recovered after treatment of the gel bands with NaOH/NaBH₄, and Bio-Gel P-4 chromatography.

*By HPAEC analysis of the acid hydrolysates of the carbohydrates isolated from SDS-polyacrylamide gel bands (from FCR-3, D6, W2, and NF54 strains) and by sensitivity to HNO₂.

*The microsequencing of the glycan core was carried out in detail using all the three analytical techniques described under "Results." The locations of the ethanolamine-phosphate and the unidentified substituents were also studied.

*The sugar sequence of the glycan core was studied only using jack bean α-mannosidase and *A. saitoi* α-mannosidase.

*Nonacetylated GlcN (Table I).

**Procedures.** Radioactive components released (50,000–60,000 cpm) were chromatographed on Bio-Gel P-4 columns (1 × 90 cm) in 100 mM pyridine, 100 mM HOAc, pH 5.2. Fractions (1.1 ml) were collected, and 60-μl aliquots were measured for radioactivity in a liquid scintillation counter. Shown are the chromatograms of the carbohydrate moieties released from the 200–215-kDa (A) and 82-kDa (B) proteins before (closed circles) and after (open circles) treatment with Pronase. C, chromatographic profiles of carbohydrate moieties released from 75-kDa (open squares), 53–56-kDa (open circles), 43–46-kDa (closed circles), and 14-kDa (closed squares) proteins. D, chromatographic profiles of carbohydrates in C after digestion with Pronase. Carbohydrate from 75-kDa (open squares), 53–56-kDa (open circles), 43–46-kDa (closed circles), 36–38-kDa (closed triangles), and 14-kDa (closed squares) proteins. Elution positions of blue dextran (V₀), sialylated glycopeptides obtained by Pronase digestion of transferrin (T), GlcNAc, and Glc are indicated.

... showed the presence of GlcN but neither sialic acid nor galactosamine. Compositional analysis of the carbohydrate moieties isolated by alkaline borohydride treatment of the major [³H]GlcN-labeled parasite proteins (from the FCR-3, D6, W2, and NF54 strains) also gave only GlcN; glucosaminol and galactosaminol were not detected (Table I). In contrast to previous reports (19–23, 28), these results demonstrate that the previously reported O-linked carbohydrates are either absent or present at very low levels in the parasite proteins.

The [³H]GlcN-labeled carbohydrate moieties of the parasite proteins (14, 36–38, 43–46, 53–56, 75, 82, and 200–215 kDa from the FCR-3 strain) released by alkaline borohydride were treated with HNO₂ and then analyzed for hexosamines (Table I). More than 90% of the GlcN was sensitive to HNO₂, indicating that the carbohydrate moieties of parasite proteins contain nonacetylated GlcN (Table I). Although unlikely, it is possible that the GlcN could have been derived from an N-acetylgalactosaminy moiety by de-N-acetylation during the treatment with alkaline borohydride. Therefore, the carbohydrate moieties of the parasite proteins released by the Pronase digestion were similarly deaminated and then partitioned between water and water-saturated 1-butanol. After deamination, ~90% of the radioactivity remained in the aqueous phase, and the remaining GlcN was easily visualized by fluorography. The gel bands were excised, and the carbohydrate moieties were isolated as described under "Experimental Procedures."

P. falciparum-infected erythrocytes were metabolically labeled with [³H]GlcN, and the parasite-infected erythrocytes were enriched by centrifugation on Percoll and then lysed. The lysates were separated on 5–20% SDS-polyacrylamide gradient gels, and the radiolabeled proteins were visualized by fluorography. The gel bands were excised, and the carbohydrate moieties were isolated as described under "Experimental Procedures."
Fig. 4. Bio-Gel P-4 chromatography of carbohydrate moieties of delipidated P. falciparum cell lysate. [3H]GlcN-labeled, delipidated, whole P. falciparum lysates were treated separately with alkaline borohydride, N-glycanase, and Pronase as described under “Experimental Procedures.” The products were analyzed on Bio-Gel P-4 (1 × 90 cm) in 0.1 M pyridine, 0.1 M HOAc, pH 5.2. Fractions (1.1 ml) were collected, and 100-μl (in the case of the first treatment) or 250-μl (the second and third treatments) aliquots were measured for radioactivity in a liquid scintillation counter. A, total carbohydrate moieties obtained by treatment with 50 mM NaOH, 1 M NaBH4. B, carbohydrate moieties released by N-glycanase. C, glycopeptides that remained in aqueous phase after Pronase digestion and partition between water and water-saturated 1-butanol. Elution positions of blue dextran (V0), sialylated glycopeptides obtained by Pronase digestion of transferrin (T), GlcNAc, and Glc are indicated.

approximately 6% of the radioactive GlcN was released from the [3H]GlcN-labeled, delipidated, whole parasite proteins by digestion with N-glycanase. Upon Bio-Gel P-4 chromatography, the released material eluted as three distinct peaks (Fig. 4B), A1, A2, and A3 representing, respectively, 1.2, 2.3, and 2.5% of the total [3H]GlcN incorporated into the parasite proteins. The eluted radioactivity was pooled as indicated in Fig. 4B and analyzed for carbohydrates. All three fractions (B1, B2, and B3) gave GlcN after acid hydrolysis. However, HPAEC analysis before acid hydrolysis, showed the absence of hexosamines and N-acetylhexosamines in fraction B3. Fraction B2 does not bind to Ag 1-8 X 12 (H+) resin, suggesting that it is not free GlcN. However, almost all radioactivity in fraction B3 was bound to Ag 1-8X (carbonate) and Ag 4-X (base) resins. These results suggest that fraction B3 is a negatively charged (possibly phosphate) derivative of GlcNAc. Fractions B2 and B3 gave both GlcN and GlcNAc on sequential Nabh4 reduction, acid hydrolysis, and N-acetylation suggesting that they contain reducing end GlcNAc. Fraction B2 appears to release 1 or 2 residues of Man on treatment with jack bean α-mannosidase. On Bio-Gel P-4, the elution position of fraction B2 corresponds to ManαGlcNAc. Together, these results suggest that parasite proteins contain a low level of low molecular weight N-linked oligosaccharides. Further characterization of these carbohydrates could not be carried out because of their low abundance.

Digestion of the [3H]GlcN-labeled parasite proteins with Pronase and partitioning of the digest between water and 1-butanol gave ~90% of the radioactivity in the organic phase and the remainder in the aqueous layer. After acid hydrolysis, the materials in both the organic and aqueous phases gave only [3H]GlcN as the radioactive sugar. The GlcN residues of these carbohydrates in the 1-butanol phase were almost quantitatively sensitive to HNO2, suggesting that this fraction represents predominantly GPI anchors. The Bio-Gel P-4 elution profile of the radioactivity in the aqueous phase is shown in Fig. 4C; the eluted radioactivity was pooled as shown. Acid hydrolysis of the fractions C1 and C2, before and after treatment with HNO2, and HPAEC analysis indicated that both fractions contained only [3H]GlcNAc; no other radiolabeled sugars were detected. Only 40–50% of the radioactivity in fraction C3 is accounted for by [3H]GlcNAc; the remainder appears to be noncarbohydrate, possibly amino acids or peptides formed from nonspecifically radiolabeled proteins due to the entry of GlcN into glycolytic pathway (53). Treatment of Fraction C3 with jack bean α-mannosidase shifted the elution position of [3H]GlcN-labeled parasite proteins on Bio-Gel P-4 to a slightly lower molecular weight region, corresponding to the removal of two or three Man residues. These results, taken together with those from alkaline β-elimination and N-glycanase treatment, suggest that parasite proteins contain a low level of N-linked oligosaccharides.

To determine whether P. falciparum also contains unsubstituted terminal residues of GlcNAc, parasites at the trophozoite and schizont stages were isolated from the Percoll-enriched infected erythrocytes by saponin treatment as described (18). In a separate experiment, [3H]GlcN-labeled parasites were released by treating the infected erythrocytes with saponin as above. SDS-PAGE and fluorography showed the presence of radiolabeled proteins in the parasites but not in the supernatant (data not shown), suggesting that the parasites were intact. The parasite lysates were galactosylated using UDP-[3H]Gal and bovine milk GalT (50). SDS-PAGE fluorography (Fig. 5) demonstrated that whereas ovalbumin and several erythrocyte proteins ranging from 20 to 300 kDa were galactosylated, none of the proteins with molecular weight >25 kDa were galactosylated in the parasite lysate. However, two distinct proteins (14.5 and 18 kDa) and a smear at 20–25 kDa were labeled in the parasite lysate. Galactosylatable proteins with comparable molecular weights were also present in the lysates of erythrocyte ghosts, albeit in low proportions (Fig. 5). Considering that the parasite feeds on erythrocyte components and that many erythrocyte glycoproteins contain high levels of terminal GlcNAc residues, it is distinctly possible that the
incubations (uninfected red cell lysates and buffer only), and the same amount of radioactivity was also eluted from control elution volume higher than the peptide). Approximately the presence of a radioactive peak corresponding to GlcNAc (at an elution volume higher than the peptide). Approximately the elution position to that of standard Man2-AHM (3.3 GU), indicating the removal of two Man residues (data not shown).

Acetylation of the neutral glycans after partial acetolysis; lanes 3 and 7, neutral glycans after treatment with jack bean α-mannosidase; lane 8, untreated neutral glycans (not shown). Taken together, these results suggest that the glycan moieties of the parasite protein GPI anchors consist of four Man residues and one GlcN residue. Digestion of the neutral glycans with A. saitoi, the 1,2-linkage-specific α-mannosidase, shifted the elution position to that of standard Man2-AHM (3.3 GU), indicating the removal of two Man residues (data not shown). Acetylation of the neutral glycans under conditions that preferentially hydrolyzes α1,6-glycosidic bonds, gave a major and a minor peak (2.4 and 1.7 GU) corresponding to that of standard Man-AHM and AHM, respectively (not shown). Treatment with jack bean α-mannosidase shifted the elution volume to that of standard AHM (not shown).

Gel filtration of neutral glycans of individual parasite protein GPI anchors on calibrated Bio-Gel P-4 columns using Glc as an internal standard, gave, in all cases, a major peak corresponding to 5.3 GU with a small amount (<5%) of higher molecular weight components; the elution positions of the major peaks were similar to the standard Man4-AHM (data not shown), suggesting that the glycan moieties consist of four hexoses and one GlcN residue. Digestion of the neutral glycans with A. saitoi, the 1,2-linkage-specific α-mannosidase, shifted the elution position to that of standard Man2-AHM (3.3 GU), indicating the removal of two Man residues (data not shown). Acetylation of the neutral glycans under conditions that preferentially hydrolyzes α1,6-glycosidic bonds, gave a major and a minor peak (2.4 and 1.7 GU) corresponding to that of standard Man-AHM and AHM, respectively (not shown). Treatment with jack bean α-mannosidase shifted the elution volume to that of standard AHM (not shown). Taken together, these results demonstrate that the glycan moieties of the parasite protein GPI anchors consist of four Man residues and one GlcN residue.

Structural Characterization of Glycans from the GPI Anchors of P. falciparum—The [3H]GlcN-labeled carbohydrate moieties, isolated by the alkaline borohydride treatment and Bio-Gel P-4 chromatography, of various parasite protein bands were separately dephosphorylated with aqueous HF and then deaminated and reduced with NaBH4. In each case, Man4-AHM was identified as the major product (5.5%) of higher susceptibility to that of authentic Man4-AHM (Fig. 6, lanes 1 and 5). Partial acetylation gave one major (Man-AHM) and two minor products (Man2-AHM and AHM) (Fig. 6, lanes 2 and 6). Digestion with jack bean α-mannosidase gave one major product.
Glycosylation of *P. falciparum* Proteins

**FIG. 7.** HPTLC analysis of partial acid hydrolysates of glycan cores from *P. falciparum* protein GPI anchors. The [3H]GlcN-labeled GPI anchors from 200–215- and 82-kDa *P. falciparum* proteins were deaminated with HNO₂ and then hydrolyzed with 0.1 M trifluoroacetic acid at 100 °C for 4 h. The partial hydrolysates were dephosphorylated with aqueous HF before and after digestion with jack bean α-mannosidase as described under "Experimental Procedures." The neutral glycans (~2000 cpm) and the products of partial acid hydrolysis (6500–8000 cpm) were analyzed on silica gel 60 HPTLC plates using the solvent system 1-propanol, acetone, water (10:6:4, v/v/v). Shown are the fluorographs of glycans derived from 82-kDa (lane 1), 200–215-kDa (lane 2) parasite protein GPI anchors. Lane 1, standard Man₄-AHM from the *T. cruzi* glycoprotein GPI anchors; lane 2, neutral glycans obtained by dephosphorylation and deamination of *P. falciparum* GPI anchors; lane 3, partial hydrolysates of the GPI anchors; lane 4, jack bean α-mannosidase-treated partial hydrolysates of the GPI anchors; lane 5, mixture of standard glycans derived from the *T. cruzi* glycoprotein GPI anchors.

The linkage position between the Man residue and the GlcN was determined by periodate oxidation. Compositional analysis using Dionex HPAEC indicated that the GlcN residues of the GPI anchors are quantitatively oxidized by periodate (data not shown). However, upon *N*-acetylation, the GlcN residues were completely resistant to periodate. These results indicate that GlcN is substituted either at C-4 or at both C-4 and C-6, but not at C-3. However, the stability of this glycosidic bond to partial hydrolysis excludes the possibility of a 1,6-linked glycosidic bond between the first Man residue and the GlcN residue. Thus, this linkage position should be 1,4.

The results of the above analyses establish the sequence Man₁–2Man₁–2Man₁–6Man₁–4AHM for the glycan cores of the parasite protein GPI anchors.

**Location of Ethanolamine-Phosphate Linkage Position**—The carbohydrate moieties of the parasite proteins (200–215 and 82 kDa) were deaminated, reduced with sodium borohydride, and then subjected to partial hydrolysis using conditions that do not affect the ethanolamine phosphate linkage (46, 49). The partial hydrolysates were divided into two equal parts. In each case, one part was directly dephosphorylated with aqueous HF and the other part was digested with jack bean α-mannosidase before dephosphorylation. The products were analyzed by gel filtration on Bio-Gel P-4 (data not shown) and by HPTLC (Fig. 7).

The partial hydrolysates that were directly dephosphorylated gave five peaks that correspond to Man₄-AHM, Man₃-AHM, Man₂-AHM, Man-AHM, and AHM on Bio-Gel P-4 chromatography (data not shown). HPTLC analysis gave a ladder of Man₄-AHM, Man₃-AHM, Man₂-AHM, Man-AHM, and AHM (Fig. 7, A and B, lanes 3). Digestion of the partial hydrolysates with jack bean α-mannosidase before treatment with aqueous HF gave a mixture of Man₄-AHM and AHM (Fig. 7, A and B, lanes 4). Man₃-AHM is formed from GPI glycan cores that were unaffected and those in which the fourth Man was cleaved during the partial hydrolysis, whereas AHM is formed from glycans in which the partial hydrolysis cleaved two or more Man residues. These results demonstrate the presence of a phosphate ester substituent on the third Man residue from AHM. This substituent is likely to be the conserved protein-anchoring ethanolamine-phosphate moiety attached to the O-6 position of the third Man residue (for review see Ref. 54).

**Evidence for the Presence of Substituents on the Terminal Man Residue**—Jack bean α-mannosidase, which removes only the unsubstituted α-Man residues from the nonreducing end, was used to identify substituents on the terminal Man residue. The GPI anchors of 200–215- and 82-kDa parasite proteins isolated by mild alkaline saponification of the Pronase-digested product or those obtained by treatment with alkaline borohydride were digested with jack bean α-mannosidase before and after treatment with HNO₂ and NaBH₄. The products were dephosphorylated with aqueous HF, and those not already deaminated were then treated with HNO₂ and NaBH₄. The GPI anchors that were treated with α-mannosidase after nitrous acid deamination gave Man₃-AHM on Bio-Gel P-4 chromatography (data not shown) and on HPTLC (Fig. 8, lanes 2 and 5). However, the GPI anchors that were digested with α-mannosidase prior to nitrous acid deamination and sodium...
borohydride reduction gave a mixture of Man₃-AHM (50–60%) and Man₄-AHM (40–50%) (data not shown, and Fig. 8, lanes 3 and 6). These results indicate that at least 50–60% of the nonreducing end Man residues are substituted and that these substituents were almost quantitatively removed under the conditions of nitrous acid treatment. The results were reproducible for several purified preparations of GPI anchors from both 200–215- and 82-kDa proteins. The GPI anchor samples, those treated and not treated with HNO₂/NaBH₄ prior to the α-mannosidase digestion, were similarly purified on Bio-Gel P-4. Therefore, the observed results were not due to incomplete removal of the terminal Man residues by jack bean α-mannosidase, caused by contaminants in samples that were not treated with HNO₂/NaBH₄. Thus, the glycan cores of the parasite protein GPI anchors have the following structure.

\[
\text{Protein–}\text{NH–CH₂–CH₂–O–P–O}^–\text{Man₁→2Man₁→6Man₁→4GlcN₁→X–Man₁}^\text{a}
\]

where X represents an unidentified substituent.

**DISCUSSION**

In this study, metabolic labeling with [³H]GlcN established that about 15 proteins of the intraerythrocytic stage *P. falciparum* are dominantly modified with carbohydrate moieties. Carbohydrate compositional analysis, partitioning of the products of Pronase digestion between water and water-saturated 1-butanol, and structural studies demonstrate that GPI anchors represent the major carbohydrate modification in these parasite proteins.

*P. falciparum* proteins contain, besides GPI anchor moieties, low levels of N-glycanase-releasable high Man type and/or incompletely processed N-linked oligosaccharides and novel negatively charged GlcNAc residues; the latter was not released by the reductive alkaline β-elimination. These structures together account for about 6% of the total [³H]GlcN incorporated into the parasite proteins. Previously, Dieckmann-Schuppert et al. (23) reported the presence of a small proportion (7–10%) of N-glycanase-releasable carbohydrates in radiolabeled parasite proteins. Although the structures were not studied (23), these were likely to be N-linked carbohydrates. Recently, Kimura et al. (28) reported the existence of N-linked carbohydrates in the parasites. Considering that GPI anchors represent the major carbohydrate modification in *P. falciparum* and that proteins are modified with GPI anchors on a mol/mol basis, the level of N-glycosylation in the parasite proteins is very low. This explains why the presence of N-glycosylation was evident only on examination of whole cell lysates and was not readily detectable in individual parasite proteins. The low content of N-linked carbohydrates in *P. falciparum* is also in agreement with the previously reported undetectable levels of dolichol pyrophosphate-oligosaccharide intermediates, and peptide N-glycosidic oligosaccharyltransferase activity in the parasite (27).

The observed low content of N-linked carbohydrates in *P. falciparum* proteins is not due to the incomplete release by N-glycanase. The amount of radiolabeled carbohydrates released by N-glycanase is comparable with the amounts of glycopeptides recovered in the water phase (~10% of the total), after partitioning of the Pronase digests of the parasite proteins between water and water-saturated 1-butanol. The carbohydrates remaining in the water phase should correspond to N-linked structures, because the GPI anchor moieties are the only other carbohydrates detectable in the parasite proteins (~90%).

In contrast to the results presented here, Kimura et al. (28) reported that the N-linked carbohydrates represent as much as 70% of the radiolabeled carbohydrates of ring and early trophozoite stage *P. falciparum*, and about 30% in old trophozoites. These investigators have not considered the presence of GPI anchor moieties in the parasite proteins. Therefore, N-linked carbohydrates and other trace amounts of glycans may have been viewed as the major constituents of the parasite proteins (28). Despite this difference, our results are in partial agreement with those of Kimura et al. regarding the structural features of N-linked carbohydrates (28). In this study, compositional analysis of the N-glycanase-released carbohydrates, after treatment with NaBH₄, gave both GlcN and glucosaminitol, suggesting that these glycans contain at least two GlcN residues, one of which is at the reducing end. Based on their size and susceptibility to jack bean α-mannosidase, these oligosaccharides appear to be Man₃-GlcNAc₆ and larger high Man type and/or hybrid type structures. Kimura et al. (28) have also found similar structures in addition to N-glycanase-released chitobiose and single residues of GlcNAc in the parasite proteins. The latter were not detected in this study. However, novel, negatively charged residues of GlcNAc apparently linked to Asn were found in this study.

The observed low content of N-glycosylation in intraerythrocytic *P. falciparum* is not due to the low abundance of potential N-glycosylation sites in the parasite proteins. The deduced amino acid sequences of erythrocytic stage *P. falciparum* proteins contain several potential N-glycosylation sites (7, 24, 26). For example, MSP-1 contains as many as 15 potential N-glycosylation sites (7), and the heat shock protein HSP-72 contains four such sites (26). Moreover, a 72-kDa C-terminal peptide of *P. falciparum* MSP-1, expressed in mammalian cells, contains one to four N-linked oligosaccharide chains per molecule.² Therefore, it is likely that the low content of N-linked carbohydrates is due to very low N-glycosylation capacity of the parasite. This conclusion is consistent with the previously reported undetectable levels of GDP-Man, dolichol pyrophosphate-oligosaccharide intermediates, and peptide N-glycosidic oligosaccharyltransferase activity (27).

In contrast to the presence of GPI anchors and N-linked carbohydrates, O-linked carbohydrates were not detectable either in the individual protein gel bands or whole *P. falciparum* lysates. In support of this conclusion, neither free N-acetylatedhexosaminilts nor oligosaccharides with N-acetylatedhexosaminilts at the reducing ends were detected after NaOH/NaBH₄ treatment of the parasite proteins radiolabeled at different stages of intraerythrocytic development. Galactosylation of lysates of *P. falciparum* free of red cells using GalT and UDP-[³H]Gal, and SDS-PAGE analysis also did not provide evidence for the abundant presence of O-linked GlcNAc residues in the parasite proteins. In contrast to these results, it was previously reported that O-glycosylation is the major carbohydrate modification in parasite proteins (20–23). The reported carbohydrate structures included single residues of O-GlcNAc (20–23) and oligosaccharides with terminal GlcNAc residues (23). While some of the discrepancies may be due to the lack of consideration given to GPI moieties (20–23, 28), others (28) appear to be due to problems with the technical approaches used (see below).

² S. Yang, D. C. Gowda, and E. A. Davidson, unpublished results.
Glycosylation of \textit{P. falciparum} Proteins

bohhydrates with \text{NaOH/NaB\textsubscript{3}H\textsubscript{4}} and for the galactosylation of terminal GlcNAc with GalT and UDP-\textsuperscript{\text{3H}}Gal. These procedures may have radiolabeled predominantly the carbohydrates of the native erythrocyte glycoproteins (23) because of the abundance of \textit{O}-linked GlcNAc and oligosaccharides bearing terminal GlcNAc in human erythrocyte glycoproteins (55). This would explain several of the previously reported contradictory results (23). First, the size and structural features of the presumed \textit{O}-linked carbohydrates of the parasite proteins were strikingly similar to those obtained from control erythrocyte proteins (23). Second, the 2.5–3 times higher level of radiolabeling of carbohydrates might have been due to batch-to-batch variations in radioactivity incorporation into the carbohydrates of the erythrocytic glycoproteins rather than radiolabeling of the parasite carbohydrates. Third, the amount of presumed Gal\textsubscript{\beta1–4}GlcNAc, obtained after galactosylation, appeared to be higher for proteins from control red cells compared with those from red cells with 10\% parasitemia. Fourth, the reported effective size of Gal\textsubscript{\beta1–4}GlcNAc on Bio-Gel P-4 was 2 GU or less (23), a figure not in agreement with the reported effective size of Gal\textsubscript{\beta1–4}GlcNAc (23). However, it has been suggested that the phosphatidylinositol transduction (35). The structural basis for this broad bioactiv-

The \textit{P. falciparum} GPI anchors can induce cytokine release in host macrophages and cause pathological conditions in mice that include transient pyrexia, hypoglycemia, and lethal cachexia (32). Recently, it has been reported that both the GPI anchors MSP-1 and MSP-2 induce nitric-oxide synthase expression in macrophages and vascular endothelial cells by a protein-tyrosine kinase-dependent and protein kinase C-de-

dependent signaling pathway (36). The GPI anchors of these proteins are also reported to up-regulate the levels of intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin expression in vascular endothelial cells as well as to cause increased leukocyte and parasite cytoadherence to vascular endothelial cells via tyrosine kinase-dependent signal transduction (35). The structural basis for this broad bioactivity of the GPI anchors of \textit{P. falciparum} proteins is not known. However, it has been suggested that the phosphatidylinositol moiety of the parasite GPI alone is not sufficient for protein-tyrosine kinase-induced cell signaling and that the glycan moiety is also involved in this activity (36).

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