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Evidence for Glycosylation Sites on the 45-Kilodalton Glycoprotein of *Mycobacterium tuberculosis*

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The occurrence of glycosylated proteins in *Mycobacterium tuberculosis* has been widely reported. However, unequivocal proof for the presence of true glycosylated amino acids within these proteins has not been demonstrated, and such evidence is essential because of the predominance of soluble lipoglycans and glycolipids in all mycobacterial extracts. We have confirmed the presence of several putative glycoproteins in subcellular fractions of *M. tuberculosis* by reaction with the lectin concanavalin A. One such product, with a molecular mass of 45 kDa, was purified from the culture filtrate. Compositional analysis demonstrated that the protein was rich in proline and that mannose, galactose, glucose, and arabinose together represented about 4% of the total mass. The 45-kDa glycoprotein was subjected to proteolytic digestion with either the Asp-N or the Glu-C endopeptidase or subtilisin, peptides were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and glycopeptides were identified by reaction with concanavalin A. Peptides were further separated, and when they were analyzed by liquid chromatography-electrospray mass spectrometry for neutral losses of hexoses (162 mass units), four peptides were identified, indicating that these were glycosylated with hexose residues. One peptide, with an average molecular mass of 1,516 atomic mass units (AMU), exhibited a loss of two hexose units. The N-terminal sequence of the 1,516-AMU glycopeptide was determined to be DPEPAPPVP, which was identical to the sequence of the amino terminus of the mature protein, DPEPAP PVPXTA. Furthermore, analysis of the glycopeptide by secondary ion mass spectrometry demonstrated that the complete sequence of the glycopeptide was DPEPAPPVPTTA. From this, it was determined that the 10th amino acid, threonine, was O-glycosidically linked to a disaccharide composed of two hexose residues, probably mannose. This report establishes that true, O-glycosylated proteins exist in mycobacteria.

Tuberculosis continues to be a major public health problem. In global terms, it is estimated that 8 million new cases and 3 million deaths occur annually (37), and this death toll is predicted to reach 3.5 million by the year 2000 (6). In order to reverse this trend, better diagnostics, vaccines, and chemotherapy must be developed in conjunction with basic research on the biology and pathogenicity of the tubercle bacilli. Towards these goals, major advances have been made in the definition of the carbohydrate and protein constituents of *Mycobacterium tuberculosis* (7, 41, 45, 60, 61) and their interaction with host cells (2, 3, 25, 48, 51). In this context, a major ill-defined topic is that of posttranslationally modified proteins.

In other biological systems, the covalent modification of proteins with acyl or glycosyl moieties is known to alter immunogenicity or pathogenicity. For example, the unique triacylated proteins of gram-negative bacteria are known to possess key biological activities such as the ability to prime cytotoxic T cells (12), activate B lymphocytes (5), and stimulate phagocytosis and cytokine production by macrophages (23). However, much of the precise information on the effects of glycosylation on protein function is derived from studies with eukaryotic glycoproteins. Ishioka et al. (32) demonstrated that protein glycosylation inhibited major histocompatibility complex presentation of individual peptides to T cells, thus altering their immunogenicity. In terms of an association of glycoproteins to virulence, the most notable examples are the surface glycoproteins of *Trypanosoma* spp. that are found to be essential for parasite survival and infectivity (34). In prokaryotes, the glycosylation of the pilin of *Neisseria meningitidis* is hypothesized to affect the adherence of these organisms to endothelial cells (56) and N glycosylation of the platelet aggregation-associated protein of *Staphylococcus sanguis* may enhance the colonization of this organism in the endocardium (13, 17). Thus, given the complexity of tuberculosis immunity and pathogenesis, it is important that the structure and function of posttranslationally modified proteins of *M. tuberculosis* be fully evaluated for their roles in such processes.

Recently, *M. tuberculosis* was reported to produce both acylated and glycosylated proteins (15, 16, 19, 60). The identification of mycobacterial lipoproteins is not surprising given the wide distribution of such structures throughout the bacterial kingdom (59). In contrast, the occurrence of bacterial glycoproteins is rare. The best characterized of the glycosylated bacterial proteins are those present in the crystalline surface layer (S layer) of archeabacteria (4). Similarly, cell surface location is a general characteristic of eubacterial glycoproteins (40, 43). Nevertheless, soluble bacterial glycoproteins have been reported for *Cellulomonas fimi* (47), *Streptococcus faecium* (35), and *Streptomyces* spp. (36). In *M. tuberculosis* or *M. bovis*, various products, mostly within the culture filtrate protein population, are defined as glycoproteins almost entirely on the basis of their ability to bind concanavalin A (ConA) (14–16) with some supporting analytical data and enzymatic data (14, 16). Discrete lectin binding is also taken to demonstrate glycosylation of a recombinant form of the 19-kDa *M. tuberculosis* membrane-associated protein expressed in *M. smegmatis* (19). Although these earlier reports clearly suggest the existence of glycosyl units on some immunologically important

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proteins of mycobacteria, the pervasiveness of carbohydrates in all mycobacterial preparations is always a source of concern (31). Thus, it was essential that rigorous chemical analyses be conducted in order to eliminate the possibility of tight noncovalent association of low-molecular-weight carbohydrates with proteins or contamination with the abundant glycolipids and lipoglycans (phosphatidylinositolmannosides [PIMs], lipoarabinomannan [LAM], and lipomannan [LM]) that populate the mycobacterial cell envelope.

This article describes the purification and chemical analyses of a ConA-reactive 45-kDa culture filtrate protein from *M. tuberculosis*. Proteolytic digestion of this protein coupled with microcapillary liquid chromatography-mass spectrometry identified several glycopeptides, and detailed analysis of one of these glycopeptides by secondary ion mass spectrometry (SI-MS) revealed a 1,516-Da peptide possessing two hexose residues. This investigation has definitively established the presence of true glycoproteins in *M. tuberculosis*, thus providing a foundation for future studies on the contribution of the glycosyl units to their immunoreactivity and role in the physiology and pathogenesis of the tubercle bacillus.

MATERIALS AND METHODS

Growth of *M. tuberculosis* and preparation of subcellular fractions. *M. tuberculosis* Erdman was cultured in 30 liters of a glycerol-alanine-salts medium (54) for 14 days with gentle agitation. The culture supernatant and cells were separated by filtration through a 0.45-μm-pore-size membrane followed by filtration through a 0.22-μm-pore-size membrane. The sterile culture filtrate subsequently was concentrated to 25 ml by ultrafiltration in the Amicon apparatus (Amicon, Inc., Beverly, Mass.) with a 10,000-molecular-weight cutoff membrane, dialyzed against 0.1 M NH_4HCO_3, frozen in a dry ice-acetone bath, and lyophilized for 24 h by using a Lyph-Lock 12 lyophilizer (Labconco Corp., Kansas City, Mo.). Cells were collected from the 0.45-μm-pore-size filters and washed twice with sterile distilled water. Subcellular fractions of the cell wall, membrane, and cytoplasm were produced as previously described (29, 41).

Purification of the 45-kDa protein. *M. tuberculosis* culture filtrate protein (134 mg) was suspended in 17 ml of 10 mM Tris-HCl (pH 8.0)–150 mM NaCl. Contaminating LAM, LM, and PIMs were removed by Triton X-114 partitioning (31). The aqueous phase of the Triton X-114 partitioning step was collected, dialyzed against 0.1 M NH_4HCO_3, and lyophilized. This lipoglycan- and lipid-free material was then suspended in a buffer containing 20 mM Tris-HCl (pH 8.4) and 8 mM trifluoroacetic acid to a final concentration of 30 mg of protein per ml and applied to a high-pressure liquid chromatography (HPLC) column (1 by 10 cm) of Protein-Pak 8HR DEAE (Waters, Milford, Mass.). Proteins were eluted with an increasing concentration of $LiClO_4$, at a flow rate of 1 ml/min by using a Waters 600E HPLC system. Fractions of 1 ml were collected. Those containing the 45-kDa protein were pooled, dialyzed against 0.1 M NH_4HCO_3, dried by lyophilization, and dissolved in a buffer consisting of 50 mM KH_2PO_4 (pH 5.75), 150 mM NaCl, and 8 mM trifluoroacetic acid, to a final concentration of 25 mg of protein per ml of buffer. Further fractionation was achieved by size exclusion chromatography on a column (1 by 30 cm) of Superose 6 and a column (1 by 30 cm) of Superose 12 (Pharmacia Biotech Inc., Piscataway, N.J.) run in tandem at a flow rate of 0.3 ml/min with a Waters 600E HPLC system. Sized fractions containing the 45-kDa protein were pooled, dialyzed against 0.1 M NH_4HCO_3, and dried. A final purification step was performed by loading 500 μg of partially purified protein on preparative 9% polyacrylamide gels (7.5 by 10 cm by 1.5 mm) run at 10 mA for 1.5 h. The resolved proteins were visualized with Coomassie brilliant blue R250, excised from the gel, and electroeluted by using a Bio-Rad 422 Electro-Eluter (Bio-Rad Laboratories, Hercules, Calif.) with 50 mM NH_4HCO_3 (24). Noncovalently associated carbohydrates were separated from the purified 45-kDa protein prior to detailed chemical analyses by reversed-phase HPLC on a C_4 column (5 cm by 4.6 mm) (Vydac, Hesperia, Calif.) attached to a Waters 600E HPLC system. The 45-kDa protein was eluted at a flow rate of 0.25 ml/min with a linear gradient of 98% solvent A (trifluoroacetic acid-water, 0.1:99.9 [vol/vol])–2% solvent B (trifluoroacetic acid-water-acetonitrile, 0.1:9.9:90 [vol/vol]) to 8% solvent A–92% solvent B.

Proteolytic digestions. The following enzymes were used for proteolytic digestion of the pure protein: endoproteinase Glu-C from *Staphylococcus aureus*, subtilisin (alkaline protease VIII) from *Bacillus subtilis*, and endoproteinase Asp-N from *Pseudomonas fragi*. All proteinases were purchased from Sigma Chemical Co. (St. Louis, Mo.). Digestions with endoproteinase Asp-N and endoproteinase Glu-C were conducted on sample quantities of 25 to 75 μg under conditions described previously (57). The digestions with subtilisin were carried out on similar quantities of protein in 0.1 M NH_4HCO_3 (pH 7.8)–1 M guanidine-HCl at 37°C for 2 h. The enzyme-to-substrate ratio was 1.5% (wt/wt) for endoproteinase Asp-N and subtilisin and 0.75% for endoproteinase Glu-C.

Peptide mapping and SI-MS. For peptide mapping, products from a subtilisin proteolytic digestion of 50 μg of purified 45-kDa protein were separated by reversed-phase HPLC using a C_{18} capillary column (530 μm by 20 cm) attached to an Applied Biosystems 10-ml syringe pump. The peptides were eluted at a flow rate of 0.02 ml/min with a linear gradient of 98% solvent A–2% solvent B to 8% solvent A–92% solvent B. A_{214} was monitored with a Shimadzu SPD-6A UV detector. An aliquot (1 μl) of individual peptide fractions was applied to a sample stage previously coated with thioglycerol as the matrix and analyzed by SI-MS to determine the molecular mass of each peptide and to screen for peptides that lost 162 atomic mass units (AMU), indicating the presence of a hexose-containing peptide. SI-MS was conducted on a TSQ-700 triple-sector quadrapole mass spectrometer (Finnigan-MAT, San Jose, Calif.) equipped with a cesium ion gun (Phrasor Scientific, Inc., Duarte, Calif.). The ion source acceleration potential was 8 kV, and the conversion diode was set to −12 kV. Calibration of the instrument was accomplished with a standard peptide of 1,818.7 AMU (53).

Microcapillary liquid chromatography-MS. Glycopeptides resulting from the digestion of the purified 45-kDa protein with subtilisin were identified by microcapillary liquid chromatography-MS (30). An aliquot (4.5 μg) of the subtilisin proteolytic digestion product was applied to a C_{18} capillary reversed-phase column (internal diameter, 250 μm) and separated as described above at a flow rate of 2 μl/min. The microcapillary liquid chromatography effluent was first monitored by UV A_{214} and then introduced directly into an on-line TSQ-700 triple-sector quadrapole mass spectrometer equipped with an electrospray ionization source operating at atmospheric pressure. The electrospray needle was operated at a voltage differential of 3 to 4 kV, with a sheath flow of methoxyethanol at 2 μl/min. N_2 was heated to 100°C for use as the drying gas. A collision gas was introduced into the mass spectrometer with an offset of −19 eV. The mass spectrometer was set to detect daughter ions that corresponded to a neutral loss of 162 AMU.

Microsequence analyses. The 45-kDa glycoprotein was resolved by two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Milford, Mass.) by electroblotting, and peptides isolated by reversed-phase HPLC were spotted directly on the polyvinylidene difluoride membrane coated with Polybrene (53). Immobilized protein and peptides were subjected to automated Edman degradation on a gas phase sequencer (26) equipped with a continuous-flow reactor (52). The phenylthiohydantoin amino acid derivatives were identified by on-line reversed-phase chromatography as described elsewhere (52).

Other analytical methods. The amino acid composition of the purified 45-kDa protein was obtained by hydrolysis of 2 μg of purified protein with 6 N HCl at 110°C for 24 h, followed by separation of amino acids by reversed-phase chromatography, ninhydrin derivitization, and detection at 570 and 440 nm with a Beckman 6300 amino acid analyzer. The internal standard was norleucine. The glycosyl content of the 45-kDa product was determined after hydrolysis with 0.5 M methanol-HCl for 12 h at 80°C. The released sugars were incubated with pyridine-acetic anhydride-methanol (1:5:50) for 15 min at 20°C to ensure N acetylation of any amino sugars and, finally, O trimethylsilylated with Tri-Sil reagent (Pierce, Rockford, Ill.) at 20°C for 30 min. The resulting trimethylsilyl derivatives were resolved by gas chromatography on a DB-1 capillary column (J & W Scientific, Folsom, Calif.) using a Hewlett-Packard model 5710 gas chromatogram fitted with a glass dropping needle (8). The internal standard was arabinitol.

PAGE. Sodium dodecyl sulfate (SDS)-PAGE was performed under reducing conditions by the method of Laemmli (39) with gels (7.5 cm by 10 cm by 0.75 mm) containing a 6% stack over a 10 or 15% resolving gel. Each gel was run at a constant current of 10 mA for 15 min followed by 15 mA for 1 h. 2D gel electrophoresis was performed on the purified 45-kDa glycoprotein by applying isoelectric focusing in tube gels containing 4% ampholytes with a pH range of 4 to 6 and 1% ampholytes with a pH range of 3 to 10 (Bio-Lyte; Bio-Rad) followed by SDS-PAGE in 15% separating gels. Proteins were visualized by staining with either Coomassie brilliant blue (11) or silver nitrate (44).

Western blot analysis. Proteins, subjected to SDS-PAGE, were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) (55) which were blocked overnight with 1% bovine serum albumin in phosphate-buffered saline, pH 7.4, containing 0.1% Tween 80. Glycosylated products were detected by using either ConA conjugated to biotin (Sigma) or ConA conjugated to peroxidase (Sigma) (20). The membranes probed with ConA-biotin were subsequently incubated with streptavidin conjugated to peroxidase (Gibco-BRL, Gaithersburg, Md.). Color development was achieved with 4-chloro-α-napthol and H_2O_2 (20).

RESULTS

Examination of subcellular fractions of *M. tuberculosis* Erdman. Young (14-day-old) cultures of *M. tuberculosis* Erdman were the source of culture filtrate, cell wall, membrane, and cytosol fractions. Approximately 8 μg of total protein from each fraction was resolved by SDS-PAGE and transferred to

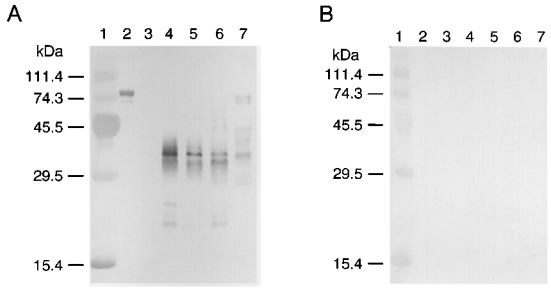

FIG. 1. Western blot analysis of *M. tuberculosis* Erdman subcellular fractions using ConA as the probe. (A) Subcellular fractions probed with ConA; (B) subcellular fractions probed with ConA in the presence of *methyl*-α-D-mannopyranoside. Lanes 1, molecular weight markers; lanes 2, positive-control glycoprotein (transferrin); lanes 3, negative-control protein (creatinase); lanes 4, cell wall; lanes 5, cell membrane; lanes 6, cytoplasm; lanes 7, culture filtrate.

nitrocellulose membranes. One membrane was probed with ConA conjugated to peroxidase (Fig. 1A), whereas a second membrane was probed with ConA-peroxidase preincubated with 250 mM *methyl*-α-D-mannopyranoside (Fig. 1B). In this fashion, several ConA-reactive products were observed in each subcellular fraction. ConA binding in the case of the cell wall, membrane, and cytosolic fractions (Fig. 1A, lanes 4 to 6) was due largely to the predominating LAM, which appeared as a diffuse spot centered at 35 kDa. Nevertheless, discrete ConA-binding products of 21, 22, 24, 33, 35, 37, 39, and 41 kDa were observed in the cell wall fraction (Fig. 1A, lane 4). A similar pattern was evident in the membrane fraction, with ConA binding observed at 21, 33, 35, 37, and 39 kDa (Fig. 1A, lane 5). The cytosolic fraction also possessed products of 21, 22, 33, and 35 kDa; however, this fraction also exhibited ConA reactivity at 28, 44, and 51 kDa (Fig. 1A, lane 6). The ConA binding profile of the culture filtrate was quite different from that of the other subcellular fractions. Unique components of 27, 40, 42, 45, and 74 kDa were apparent (Fig. 1A, lane 7). Additionally, a dominant product at 35 kDa was present; however, it was difficult to discern if this was the same product as that observed in the other fractions. The binding of ConA to each of these was readily inhibited with *methyl*-α-D-mannopyranoside (Fig. 1B), suggesting that binding was carbohydrate specific (20). The 21-kDa product in the cell wall, membrane, and cytosolic fractions was probably the 19-kDa protein originally characterized by Young and colleagues (19). Espitia and colleagues (14, 15) previously described the presence of the 38- and 55/50-kDa ConA-reactive proteins in a culture filtrate from *M. tuberculosis*, most likely corresponding to the products resolved at 35, 42, and 45 kDa in Fig. 1A, lane 7. However, they did not report a 74-kDa ConA-reactive band or the less apparent products at 27 and 40 kDa. In view of the physiological and immunological importance of the culture filtrate proteins of *M. tuberculosis* and the fact that the 45- and 74-kDa bands were observed exclusively as dominant secreted products, these two were subjected to further purification and biochemical analyses.

Purification of the 45-kDa culture filtrate protein. All mycobacterial fractions contain, to various extents, the pervasive lipoglycans (LAM, LM, and PIMs). Thus, prior to detailed biochemical analyses of glycoproteins of mycobacterial origin, it was imperative to remove these contaminants. To accomplish this, 134 mg of total culture filtrate protein was subjected to Triton X-114 partitioning, a procedure previously demonstrated to effectively remove the majority of mycobacterial lipoglycans and glycolipids from hydrophilic proteins (31). The

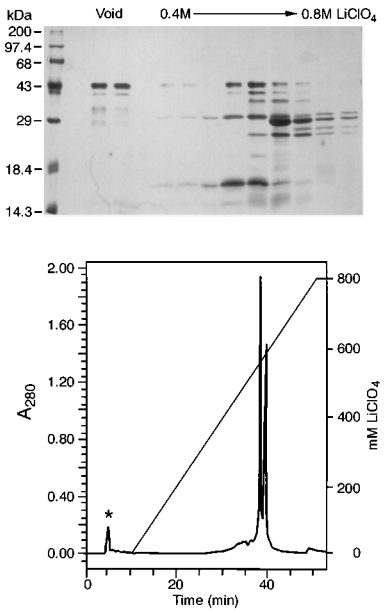

FIG. 2. Fractionation of *M. tuberculosis* Erdman culture filtrate proteins by DEAE HPLC. The silver nitrate-stained SDS-polyacrylamide gel shows every fraction collected from 2 to 6 min, representing the void volume, and every other fraction collected from 33 to 49 min, representing those proteins eluted with 0.4 to 0.8 M $LiClO_4$. The corresponding protein elution profile of the culture filtrate fractionated by DEAE HPLC, and monitored by A_{280}, is shown below. *, elution of the 45-kDa glycoprotein.

aqueous fraction of this partitioning step, containing 96 mg of protein, was further fractionated by anion-exchange chromatography. The void material from a DEAE HPLC column was largely composed of the 45-kDa product (Fig. 2). Examination of this material by Western blot analysis, with ConA-peroxidase as the probe, demonstrated the presence of a glycosylated product migrating at 45 kDa (Fig. 3). However, the same Western blot analysis unexpectedly revealed that the 74-kDa product was also present in the voided material (Fig. 3). The inability to detect the 74-kDa product with either silver nitrate or Coomassie blue (Fig. 2), in contrast to its clear reactivity to ConA (Fig. 3), implied that this component was not protein in nature but more likely polysaccharide or lipoglycan (42). Thus,

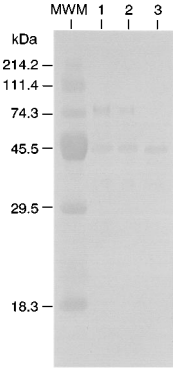

FIG. 3. ConA-Western blot analysis of the partially purified and fully purified 45-kDa glycoprotein obtained from each purification step. Lane 1, the fraction enriched in the 45-kDa protein obtained by DEAE HPLC; lane 2, the partially purified protein resulting from size exclusion chromatography; lane 3, purified 45-kDa protein obtained by preparative PAGE and electroelution. MWM, molecular weight markers.

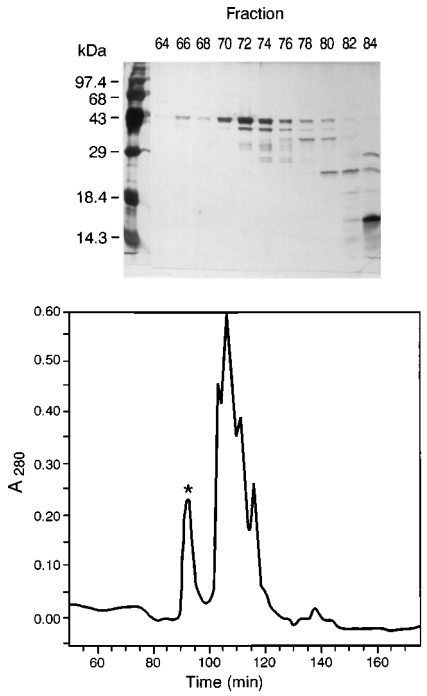

FIG. 4. Fractionation by size exclusion chromatography of proteins included in the void fraction obtained by DEAE chromatography. The silver nitrate-stained SDS-polyacrylamide gel of every other fraction from fraction 64 to 84 corresponds to the 90- to 120-min segment of the protein elution profile obtained by monitoring A_{280}. The 92-min peak (*) is represented by fractions 64 to 70 and corresponds to the partially purified 45-kDa glycoprotein.

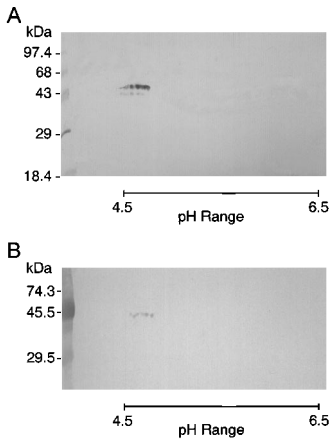

FIG. 5. 2D PAGE and 2D Western blot analysis of the purified 45-kDa glycoprotein. (A) Silver nitrate-stained 2D polyacrylamide gel of the purified 45-kDa product (2.5 μg); (B) 2D Western blot analysis of the purified 45-kDa product (2.5 μg) using ConA-peroxidase as the probe. The observed pI was determined by corunning the purified glycoprotein with 2D PAGE standards (Bio-Rad Laboratories, Hercules, Calif.).

subsequent efforts were directed to purification and characterization of the 45-kDa product. The pooled void volume from the DEAE column containing 26 mg of protein was fractionated by size exclusion chromatography. The 45-kDa product was obtained in a relatively pure form in fractions 64 to 70 from the Superose 6 and Superose 12 columns run in tandem (Fig. 4). Only minor contamination with a 42-kDa protein was observed by SDS-PAGE and silver nitrate staining. ConA-Western blot analysis again confirmed that the 45-kDa protein was glycosylated (Fig. 3). However, this Western blot analysis also demonstrated that the 45-kDa protein was still contaminated with the 74-kDa product. A final purification step was performed by preparative SDS-PAGE and electroelution, yielding 1.6 mg of the purified 45-kDa glycoprotein. Western blot analysis confirmed that the 74-kDa material had been removed (Fig. 3). The purity of the 45-kDa product was confirmed by 2D PAGE and 2D PAGE–ConA-Western blot analysis (Fig. 5). These analyses demonstrated that the purified 45-kDa protein, with a pI centered at 4.6, was devoid of observable lipoglycans or other proteins. The monoclonal antibody 6A3, generated against the previously described 55/50-kDa *M. tuberculosis* glycoprotein (14), was found to be reactive to the purified 45-kDa culture filtrate protein by Western blot analysis (data not shown). Additionally, polyclonal serum generated against MPT 32, a 43-kDa culture filtrate protein originally identified by Nagai and colleagues (45), was also reactive with the 45-kDa protein (data not shown).

Compositional analysis of the 45-kDa protein. Prior to amino acid and carbohydrate analysis, the 45-kDa protein was subjected to a final purification step by reversed-phase chromatography. Carbohydrate analysis by means of trimethylsilyl derivitization and gas chromatography demonstrated that 3.8 nmol of mannose, 2.43 nmol of glucose, 2.24 nmol of galactose, and 1.86 nmol of arabinose were associated with 1 nmol of the purified 45-kDa protein. The derivitization of the released sugars included a step for the N acetylation of amino sugars to ensure their detection; however, none were found. Overall, carbohydrate was estimated to constitute about 4% (wt/wt) of the 45-kDa product, a value much less than the 34.6% previously reported (15). Seemingly, the additional reversed-phase chromatography step was crucial in removing contaminating lipoglycans, since analysis of carbohydrate content of the 45 kDa protein prior to reversed-phase chromatography yielded a value of 23.6%. Amino acid analysis demonstrated that the 45-kDa protein was particularly rich in proline and alanine; these two amino acids accounted for 38.0% of the total amino acids (Table 1).

Analysis of proteolytic digests. Previous work has relied on lectin binding or carbohydrate analysis to demonstrate the existence of glycoproteins in mycobacteria. Both methods are

TABLE 1. Amino acid composition of the 45-kDa glycoprotein[a]

Amino acid	pmol	mol%
Pro	500.1	19.0
Ala	484.3	19.0
Asx	279.1	12.0
Glx	257.9	10.0
Ser	180.0	7.70
Leu	166.6	6.40
Thr	189.9	6.20
Val	159.1	6.20
Lys	96.8	3.70
Arg	78.5	3.10
Ile	77.2	3.00
Phe	62.5	2.40
Tyr	31.4	1.20
His	29.4	1.10
Gly	11.3	0.50

[a] Amino acids were released by acid hydrolysis of 20 pmol of the purified 45-kDa glycoprotein, separated by reverse-phase chromatography on a C_{18} column, derivatized with ninhydrin, and quantitated colorimetrically by using a Beckman 6300 amino acid analyzer. Norleucine was included as the reference peak. Cysteine, methionine, and tryptophan cannot be assayed by this method.

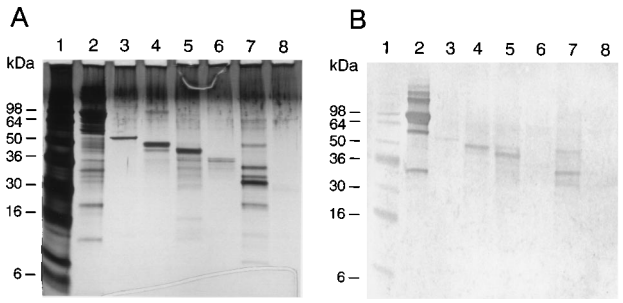

FIG. 6. SDS-PAGE and ConA-Western blot analysis of enzymatic digests of the purified 45-kDa glycoprotein. (A) Silver nitrate-stained Tricine polyacrylamide (16%) gel of the digested 45-kDa glycoprotein; (B) ConA-Western blot of the digested 45-kDa glycoprotein. Lanes 1, molecular weight markers; lanes 2, positive-control glycoprotein (transferrin); lanes 3, negative-control protein (creatinase); lanes 4, untreated 45-kDa glycoprotein; lanes 5, digest of the 45-kDa glycoprotein with endoproteinase Glu-C; lanes 6, endoproteinase Glu-C; lanes 7, digest of the 45-kDa glycoprotein with endoproteinase Asp-N; lanes 8, endoproteinase Asp-N.

valid for the detection of carbohydrates; however, they are less than critical in distinguishing between covalently and noncovalently associated glycosyl residues. In order to generate more definitive evidence for true glycoproteins, the 45-kDa protein was digested with the Glu-C or Asp-N endoproteinase and the products were resolved on a Tricine-polyacrylamide gel, transferred to nitrocellulose, and probed with ConA-peroxidase. Both the Glu-C and the Asp-N endoproteinase digestions (Fig. 6A, lanes 5 and 7) yielded several degradation products that were of lower molecular weight than the purified 45-kDa protein (Fig. 6, lanes 4). The digestion with Glu-C produced a dominant 42-kDa peptide and several minor peptides ranging from 27 to 8 kDa (Fig. 6A, lane 5). Additionally, products of 36 to 30 kDa were observed with the Glu-C-digested material; however, the same peptide fragments were also observed for the Glu-C endoproteinase alone (Fig. 6A, lane 6). Of the peptide fragments produced by digestion with Glu-C, only the 42-kDa fragment reacted with ConA-peroxidase (Fig. 6B, lane 5). Digestion of the 45-kDa protein with Asp-N resulted in peptide fragments of 34, 32, 31, 21, 13, and 9 kDa (Fig. 6A, lane 7), of which only the 34-kDa product showed strong ConA reactivity (Fig. 6B, lane 7). The fact that a single peptide from each proteolytic digestion reacted with the ConA-peroxidase indicated that the glycosyl residues were covalently associated. Nonetheless, it was arguable that the size and complexity of these ConA-binding fragments would allow strong noncovalent interactions with glycosyl residues. Thus, the more definitive method of microcapillary HPLC coupled to electrospray MS was applied. Peptides resulting from subtilisin digestion were resolved by reverse-phase HPLC, and individual glycopeptides were identified by electrospray MS neutral-loss scanning for products that fragmented with an *m/z* difference of 162, the ion which is diagnostic for the fragmentation of hexose residues from the parent ion (30). The subtilisin digest yielded 18 peptides, S_1 to S_{18} (Fig. 7A), and, of these, four peptides (S_1, S_3, S_6, and S_{11}) produced *m/z* differences of 162 (Fig. 7B). Only the S_{11} peptide was obtained in sufficient amount and purity to be analyzed further. N-terminal amino acid analysis demonstrated the sequence DPEPAPPVP for S_{11}, which was identical to the first 9 amino acids of the intact 45-kDa protein, DPEPAPPVPXTA (note that the 10th amino acid in this sequence was not identified). The S_{11} peptide was then subjected to analysis by SI-MS under mild ionization conditions, such that fragmentation of glycosyl residues rather than of the peptide was achieved. A pseudomolecular ion $(M + H)^+$ of *m/z* 1,516

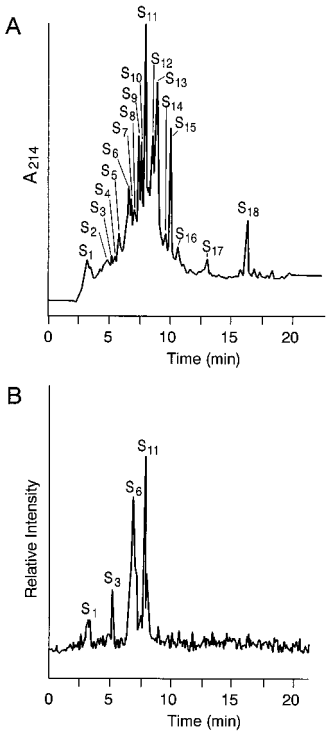

FIG. 7. Liquid chromatography-MS of the 45-kDa glycoprotein digested with subtilisin (alkaline protease VIII). Peptide fragments were resolved by C_{18} capillary reversed-phase HPLC. (A) Elution profile of peptides detected by A_{214}; (B) peptides detected by electrospray MS that fragmented with a neutral loss of 162 AMU.

and fragment ions of *m/z* 1,354 and 1,192 were obtained (Fig. 8), pointing to sequential loss of one and two hexose units from the S_{11} peptide. The *m/z* 1,192 fragmentation ion, i.e., the fully deglycosylated peptide, was 273 mass units greater than the $(M + H)^+$ molecular mass calculated for the 9-amino-acid peptide (DPEPAPPVP) determined by N-terminal sequencing of the

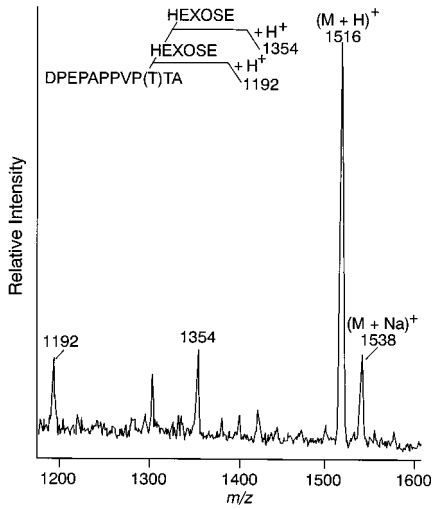

FIG. 8. SI-MS of the S_{11} peptide generated from the subtilisin digestion of the purified 45-kDa glycoprotein. The amino acid sequence of the S_{11} peptide as shown, was determined by N-terminal amino acid sequencing of the purified 45-kDa glycoprotein and the S_{11} peptide.

S_{11} peptide. This is to be compared with the calculated $(M + H)^+$ molecular mass (1,192 AMU) for the peptide defined by N-terminal amino acid sequencing of the intact 45-kDa protein (DPEPAPPVPXTA) if threonine is substituted for the unknown amino acid at position 10. Thus, the inability to obtain a complete amino acid sequence for the S_{11} peptide and to define the amino acid residue at position 10 is due to the presence of *O*-glycosyl residues at the X/Thr position, a conclusion supported by the literature (1, 22). Further evidence for O glycosylation of the 45-kDa protein was provided by the ready release of the hexoses from this protein by alkaline catalyzed β-elimination (18) (data not shown). The hexoses on peptide S_{11} were also susceptible to jack bean α-D-mannosidase, indicating that the unit was probably a mannobiose. The combined results demonstrated the amino acid sequence of the S_{11} peptide to be DPEPAPPVPTTA, in which the first threonine residue is O glycosylated with a disaccharide composed of two mannose units.

DISCUSSION

Daniel and colleagues were probably the first to actively pursue the concept of glycosylated proteins in *M. tuberculosis*. A series of monoclonal antibody binding studies of antigen 5 (the 38-kDa PhoS homolog) suggested that it shares epitopes with lipoarabinomannan (10, 46), a finding which was taken to imply the presence of a carbohydrate "intimately associated" with the protein molecule. Subsequently, analysis of highly purified antigen 5 by quantitative gas chromatography of alditol acetates, conducted by one of us (P. J. Brennan [with S. W. Hunter]) on behalf of T. M. Daniel, revealed a total carbohydrate content of 11.6%, composed mostly of glucose, mannose, and arabinose, leading to the conclusion that "the carbohydrate component of antigen 5 preparations is due to minor, nondescript contamination" rather than specific glycosylation (9). However, in the light of subsequent work, this conclusion may not have been justified.

Espitia and Mancilla (15) have since identified three ConA-binding proteins (bands) of 55, 50, and 38 kDa within the culture filtrate protein of *M. tuberculosis*. In subsequent studies Espitia et al. (14) provided additional evidence for glycosylation of the 55/50 kDa protein complex by demonstrating that treatment of this protein with jack bean α-D-mannosidase resulted in a loss of ConA binding and a shift in the migration of this protein in SDS-PAGE. Fifis et al. (16) demonstrated that the 38-, 25-, 22-, and 19-kDa proteins from the culture filtrate of *M. bovis* did contain carbohydrate, again on the basis of ConA binding, supported by chemical analysis. Garbe et al. (19) demonstrated in a similar fashion that the 19-kDa protein, already known to contain a signal peptide sequence and an acylation consensus sequence, was glycosylated when expressed as a recombinant protein in *M. smegmatis*. In these more recent studies, it appeared unlikely that contamination with the ubiquitous lipoglycans or phosphoglycolipids (LAM, LM, and PIMs) was responsible for the apparent glycosylation of proteins. However, conscious of the pervasiveness of these products in mycobacterial extracts, we sought more definitive evidence.

The present work utilized primarily SI-MS to chemically prove that a peptide fragment derived by proteolytic digestion of one of the ConA-reactive bands from the culture filtrate of *M. tuberculosis* was covalently glycosylated. The results clearly demonstrate that the 45-kDa protein is O glycosylated and, specifically, that the S_{11} glycopeptide is O glycosylated at a threonine residue. Unlike sites of N glycosylation, strict consensus sequences for O glycosylation of proteins are not apparent (21). Nevertheless, information derived from an examination of eukaryotic glycoproteins has yielded several potential motifs for O glycosylation (22, 58), the most common of which is serine or threonine within proline-rich domains (27). Moreover, glycosylation of this serine or threonine residue is increased significantly if a proline residue is located at positions −1 or +3 relative to it as a site of O glycosylation (58). Indeed, examination of the literature revealed that cellulase produced by *Cellulomonas fimi*, the only other known eubacterial glycoprotein O glycosylated at a threonine residue, contains a proline-rich domain (47). Interestingly, this type of motif is present at the proposed site of O glycosylation in the S_{11} glycopeptide.

Proteolytic digestion of the 45-kDa protein with subtilisin resulted in the formation of at least four glycopeptides. However, given the different motifs described for O glycosylation, it is not possible to predict whether the other glycopeptides will contain motifs similar to the one present on the S_{11} peptide. A universal structure-function relationship for the glycosylation of proteins is not known. Nonetheless, important biological functions have been attributed to O glycosylation. Of the proteins included in the S-layer of archaebacteria, several are O glycosylated (4, 40, 43), and this modification is proposed to play a direct role in the structure of the S-layer and in the ability of these organisms to survive in hostile environments (4, 40). Others have noted that protein O glycosylation is typically clustered in heavily glycosylated domains and that protein folding within these domains is limited, leading to an extended conformation (33). Another attributed function of O glycosylation is the enhancement of protein export (38), and, although little is known about the mechanism of protein export across the extensive mycobacterial cellular envelope, it is tempting to speculate that protein glycosylation plays a central role in the secretion of specific mycobacterial proteins. A final hypothesis is that glycosylation of the 45-kDa glycoprotein increases the stability of this protein in the intracellular environment in which *M. tuberculosis* normally resides, an idea supported by the fact that O-glycosylated proteins are more resistant to proteolytic digestion (33). Several studies also demonstrate that glycosylated proteins are key to aspects of the immunogenicity and pathogenicity of the host organism (32, 34, 56). However, without a defined biological role for the 45-kDa glycoprotein, the purpose of its glycosylation cannot be assessed at this time.

The N-terminal amino acid sequence of the 45-kDa glycoprotein is 92% homologous with MPT 32 (45) and 75% homologous to the 55/50-kDa protein described by Espitia et al. (14). This homology of N-terminal sequences and the reactivity of the 45-kDa glycoprotein to the monoclonal antibody 6A3 (14) and polyclonal serum generated against MPT 32 (45) indicate that these three independently described *M. tuberculosis* culture filtrate proteins are the same. The discrepancy in the N-terminal sequences is most likely due to the fact that this region of the protein is glycosylated and rich in proline. Both of these factors are known to present obstacles in acquiring accurate sequence data by Edman degradation (1, 28). The N-terminal sequence of the 45-kDa glycoprotein also demonstrates 75% homology to that of a 47/45 kDa protein isolated from the culture filtrate of *M. bovis* by Romain and colleagues (50). This 47/45-kDa protein was reported to be reactive to sera from animals experimentally infected with live but not killed *M. bovis* (50). Thus, if the *M. bovis* 47/45-kDa protein is the homolog of the *M. tuberculosis* 45-kDa glycoprotein, then the selective reactivity to immune sera may be explained by the fact that this product is a true excreted protein, absent from other subcellular fractions. It has been shown that a 50- to

55-kDa proline-rich protein from *M. bovis* BCG is capable of differentiating animals experimentally infected with live, as distinct from killed, organisms when used as a skin test antigen (49). However, the N-terminal amino acid sequence of the *M. tuberculosis* 45-kDa glycoprotein described in this report is only 25% homologous to that of the *M. bovis* 50- to 55-kDa protein. Further chemical characterization of this 45-kDa glycoprotein in addition to sequencing of the corresponding gene will demonstrate its relationship to other reported proteins, allow for the structural elucidation of the oligosaccharide substituents and determination of the sites and extent of additional glycosylation, and provide insight into the function of this protein.

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