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Definition of the Full Extent of Glycosylation of the 45-Kilodalton Glycoprotein of *Mycobacterium tuberculosis*

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Chemical evidence for the true glycosylation of mycobacterial proteins was recently provided in the context of the 45-kDa MPT 32 secreted protein of *Mycobacterium tuberculosis* (K. Dobos, K. Swiderek, K.-H. Khoo, P. J. Brennan, and J. T. Belisle, Infect. Immun. 63:2846–2853, 1995). However, the full extent and nature of glycosylation as well as the location of glycosylated amino acids remained undefined. First, to examine the nature of the covalently attached sugars, the 45-kDa protein was obtained from cells metabolically labeled with D-[U-^{14}C]glucose and subjected to compositional analysis, which revealed mannose as the only covalently bound sugar. Digestion of the protein with the endoproteinase subtilisin and analysis of products by liquid chromatography-electrospray-mass spectrometry on the basis of fragments demonstrating neutral losses of hexose (*m*/*z* 162) or pentose (*m*/*z* 132) revealed five glycopeptides, S_7, S_{18}, S_{22}, S_{29}, and S_{41}, among a total of 50 peptides, all of which produced only *m*/*z* 162 fragmentation ion deletions. Fast atom bombardment-mass spectrometry, N-terminal amino acid sequencing, and α-mannosidase digestion demonstrated universal O glycosylation of Thr residues with a single α-D-Man, mannobiose, or mannotriose unit. Linkages within the mannobiose and mannotriose were all α1-2, as proven by gas chromatography-mass spectrometry of oligosaccharides released by β-elimination. Total sequences of many of the glycosylated and nonglycosylated peptides combined with published information on the deduced amino acid sequence of the entire 45-kDa protein demonstrated that the sites of glycosylation were located in Pro-rich domains near the N terminus and C terminus of the polypeptide backbone. Specifically, the Thr residues at positions 10 and 18 were substituted with α-D-Man*p*(1→2)α-D-Man*p*, the Thr residue at position 27 was substituted with a single α-D-Man*p*, and Thr-277 was substituted with either α-D-Man*p*, α-D-Man*p*(1→2)α-D-Man*p*, or α-D-Man*p*(1→2)α-D-Man*p*(1→2)α-D-Man*p*. This report further corroborates the existence of true prokaryotic glycoproteins, defines the complete structure of a mycobacterial mannoprotein and the first complete structure of a mannosylated mycobacterial protein, and establishes the principles for the study of other mycobacterial glycoproteins.

Initially, the occurrence of bona fide glycosylated proteins in eubacteria was difficult to accept. Now, as a result of the application of critical analytical techniques, the presence of such molecules in such eubacteria as *Neisseria meningitidis* (43), *Flavobacterium meningosepticum* (36, 37), *Streptococcus sanguis* (11), *Bacillus alvei* (32), *Clostridium* spp. (15, 31), *Bacteroides cellulosolvens* (15), *Thermoanaerobacter thermohydrosulfuricus* (3), and *Mycobacterium tuberculosis* (10) is recognized. In the case of *M. tuberculosis*, full definition of glycosylation sites and the nature and extent of glycosylation are lacking. Initial evidence for the presence of glycoproteins in *M. tuberculosis* was based on the observation of discrete concanavalin A-binding products upon polyacrylamide gel electrophoresis (PAGE) and electroblotting of protein preparations (13, 14). However, since these patterns occurred in the midst of considerable quantities of mannose (Man)-containing lipoglycans and phospholipids (10), chemical proof of amino acid glycosylation was considered necessary.

The 45-kDa culture filtrate protein of *M. tuberculosis* is one case in point. Recent antibody reactivity studies and N-terminal amino acid sequencing conducted by both Espitia et al. (12, 13) and Dobos et al. (10) demonstrated that this 45-kDa protein is the same as MPT 32, a culture filtrate protein originally isolated by Nagai and colleagues (34). Also, more recently, the DNA sequence of a gene designated *apa* encoding a 45/47-kDa *M. tuberculosis* protein was elucidated by Laqueyrerie et al. (27), and the deduced amino acid sequence of this gene yielded 100% homology with the N-terminal sequence of the 45-kDa MPT 32 protein (10). The *apa* sequence further indicated that this protein is rich in Pro and Ala, confirming earlier amino acid compositional analysis (10, 12).

Dobos et al. (10), like others before (13), concluded that the 45-kDa protein was glycosylated, on the basis of concanavalin A-binding patterns, but deliberately sought more definitive chemical proof. Among the products of proteolysis was a glycopeptide with an average molecular mass of 1,516 atomic mass units (AMU) (10), which was shown to be O glycosylated at a Thr site with two mannose residues (10), and, in addition, the glycosylated Thr was the first of a pair of Thr residues situated at the positions 10 and 11 from the amino terminus of the mature glycoprotein, i.e.,

DPEPAPPVPTTA-
|
Man-Man

The presence of other glycopeptides was also recognized by Dobos et al. (10), but they were not further characterized. This current work describes the purification and chemical characterization of five glycopeptides from proteolytic digests of the 45-kDa MPT 32 glycoprotein. N-terminal amino acid sequencing coupled with fast atom bombardment-mass spectrometry

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(FAB-MS) demonstrates that each contains O-glycosylated Thr residues within a newly recognized, conserved O-glycosylation motif. Carbohydrate and MS analysis established that the glycosylation units in each case consist of single mannose, mannobiose, or mannotriose units possessing α1-2 linkages. Also, the recent elucidation of the complete gene sequence for this protein (27) now allows the location of all glycosylation sites within the N- and C-terminal regions of the polypeptide backbone. Moreover, the elucidation of the complete primary structure of this unique glycoprotein enables speculation and future research, on its biosynthesis and physiological role, complementary to ongoing work on its immunogenicity (38, 39).

MATERIALS AND METHODS

Growth and metabolic labeling of *M. tuberculosis* with [U-^{14}C]glucose. *M. tuberculosis* Erdman was cultured in glycerol alanine salts medium (46) for 14 days at 37°C with gentle agitation, conditions considered optimal for the production of culture filtrate proteins including the 45-kDa protein. To obtain the radiolabeled glycoprotein, *M. tuberculosis* was grown in 5 liters of the medium at 37°C with gentle agitation for 5 days, at which time D-[U-^{14}C]glucose (3 mCi/mmol; American Radiolabeled Chemicals, Inc., St. Louis, Mo.) was added to a final concentration of 1 mCi/liter, and the culture was incubated for an additional 8 days. The ^{14}C-labeled culture filtrate proteins were harvested as described previously (10).

Purification of the 45-kDa glycoprotein. The protocol described previously was applied with modifications (10). The culture filtrate was extracted with Triton X-114 (22), and the aqueous phase was applied to a high-pressure liquid chromatography (HPLC) column (1 by 10 cm) of Protein-Pak 8HR DEAE (Waters, Milford, Mass.) connected to a Waters 600E HPLC system. The proteins were eluted with a gradient of $LiClO_4$ as described previously (10). Fractions containing the 45-kDa glycoprotein were identified by sodium dodecyl sulfate (SDS)-PAGE (26, 33) and immunoblot analysis (48) using anti-MPT32 polyclonal serum (provided by Sadman Nagai) as the probe. Fractions were pooled, dialyzed against 0.1 M NH_4HCO_3, dried by lyophilization, dissolved in solvent A (trifluoroacetic acid-water, 0.1:99.9), and applied to a reversed-phase HPLC column (25 by 1.0 cm) of diphenyl-modified silica (Vydac, Hesperia, Calif.) connected to a Waters 600E HPLC system. The 45-kDa protein was eluted at a flow rate of 2 ml/min with a linear gradient of 80% solvent A–20% solvent B (trifluoroacetic acid–*n*-propanol–water, 0.1:90:9.9) to 15% solvent A–85% solvent B. Fractions (1 ml) containing the purified 45-kDa glycoprotein were again identified by SDS-PAGE and immunoblot procedures. To ensure that all noncovalently associated carbohydrates were removed from the purified, radiolabeled 45-kDa glycoprotein, the material was rechromatographed by use of the reversed-phase HPLC approach.

Enzymatic digestions and peptide mapping. The purified 45-kDa glycoprotein (500 μg) was digested with either subtilisin (alkaline protease VIII; Sigma Chemical Co., St. Louis, Mo.) or a mixture of chymotrypsin-trypsin (1:1; Sigma). The proteolytic digestions were carried out in 0.1 M NH_4HCO_3 (pH 7.8)–1 M guanidine-HCl at 37°C for 2 h (10, 49). Products from both digestions were separated by reversed-phase HPLC on a column (4.6 by 250 mm) of C_{18} (Vydac). The peptides resulting from digestion with subtilisin were eluted at a flow rate of 0.5 ml/min with a multistep, linear gradient of 90% solvent A and 10% solvent C (trifluoroacetic acid-acetonitrile-water, 0.1:90:9.9) to 45% solvent A–55% solvent C over 60 min, followed by a linear gradient of 45% solvent A–55% solvent C to 8% solvent A–92% solvent C over 20 min. The peptides generated from the chymotrypsin-trypsin digest were eluted by use of a linear gradient of 98% solvent A–2% solvent C to 8% solvent A–92% solvent C over a 60-min period. The elution of peptides was monitored by A_{214} with a Waters 486 UV detector.

Digestion of the protein with α-mannosidase was conducted as follows. The radiolabeled 45-kDa glycoprotein (135 μg) or purified glycopeptides (320 ng to 3 μg) were solubilized in 100 μl of 0.05 M CH_3COONa (pH 4.5) and incubated with 10 μl of α-mannosidase from *Canavalia ensiformis*, supplied as a 5-mg/ml suspension (Boehringer Mannheim, Indianapolis, Ind.), at 37°C for 8 h. An additional 10 μl of α-mannosidase was added to the reaction mixture, and the mixture was incubated for an additional 16 h at 37°C (23). The digestions were terminated by incubating at 100°C for 2 min, dried, and suspended in 5% CH_3COOH. The released Man residues were separated from the 45-kDa protein by applying the products to a C_{18} reversed-phase Sep-Pak cartridge (Waters) and washing with 5% CH_3COOH, followed by elution of the α-mannosidase-digested protein with *n*-propanol–acetic acid–water (50:5:45). The α-mannosidase-digested glycopeptides were separated from the released sugars in a similar manner. After washing the Sep-Pak cartridges with 5% CH_3COOH, the peptides were eluted with *n*-propanol–acetic acid–water (25:5:70) followed by *n*-propanol–acetic acid–water (50:5:45).

Carbohydrate analysis. For purposes of analyzing the sugar components of the protein, the radiolabeled 45-kDa protein (100 μg) was hydrolyzed with 2 M trifluoroacetic acid at 120°C for 2 h. The hydrolyzed material was dried under nitrogen, solubilized in water, and applied to an HPLC column (4 by 250 mm) of CarboPac PA1 (Dionex Corp., Sunnyvale, Calif.) connected to a Dionex HPLC system equipped with an advanced gradient pump and a pulsed amperometric detector. Monosaccharides were eluted from the column with 10 mM NaOH at a flow rate of 1 ml/min (9), collected in 1-ml fractions, and counted by liquid scintillation, and the elution profile was compared with that of a mixture of known monosaccharides.

Mass spectral analysis of peptides and glycopeptides. The products from subtilisin and chymotrypsin-trypsin digestions of the 45-kDa glycoprotein were resolved by microcapillary C_{18} reversed-phase HPLC (45). Effluent was monitored by A_{214} and then introduced directly into an on-line TSQ-700 triple-sector quadrapole MS with an electrospray ionization source and collision gas as described previously (45). Glycopeptides were detected by scanning for a neutral loss of 162 AMU, indicative of the loss of hexose (i.e., Man) units, or 132 AMU for the loss of pentose units (21).

Methyl esterification of selected α-mannosidase-digested peptides was performed in 0.5 N methanolic HCl at 20°C for 30 min. Upon drying under N_2, the methyl-esterified peptides were N-acetylated in methanol-pyridine-acetic anhydride (50:1:5) for 30 min at 20°C.

The molecular weights of peptides, glycopeptides, and α-mannosidase-digested peptides were determined by FAB-MS. Individual peptides were dissolved in 5% CH_3COOH (10 to 20 μl), and samples (1 μl) were added to the thioglycerol matrix. FAB-MS was conducted on a VG Autospec (Fission Instruments, Inc., Beverly, Mass.) fitted with a cesium ion gun operated at 25 to 30 kV. Data acquisition and processing were performed with VG Analytical Opus software (Fission Instruments).

Preparation and analysis of oligoglycosyl alditols. Oligosaccharides were released from purified glycopeptides by reductive β-elimination conducted as follows (6). Glycopeptides (320 ng to 3 μg) were suspended in a solution of 0.05 M NaOH containing 1 M $NaBH_4$ and maintained at 37°C for 4 h. The reactions were neutralized and desalted by the addition of Dowex 50W × 8 (H^+) beads (Sigma). Supernatants were collected and repeatedly evaporated to dryness under a stream of N_2 with the addition of 10% CH_3COOH in CH_3OH. The released oligoglycosyl alditols were permethylated (8), hydrolyzed with 2 M CF_3COOH, reduced with $NaB[^2H]_4$, and peracetylated (30). Gas chromatography (GC)-MS of the partially methylated alditol acetates was performed on a Hewlett-Packard 5890 gas chromatograph fitted with a DB-5 capillary column (15 m by 0.25 mm [inside diameter]; J & W Scientific, Folsom, Calif.) and connected to a Hewlett-Packard 5790 mass detector as described previously (30).

Microsequence analysis. Peptides isolated by reversed-phase HPLC or recovered following digestion with α-mannosidase were spotted directly onto a polyvinylidene difluoride membrane precoated with polybrene as described before (45). Immobilized peptides were subjected to automated Edman degradation on a gas-phase sequencer (42) equipped with a continuous-flow reactor (19). The phenylthiohydantoin amino acid derivatives were identified by on-line reversed-phase chromatography (45).

RESULTS

Sugar analysis of the ^{14}C-labeled 45-kDa glycoprotein. Previous compositional analyses of the 45-kDa glycoprotein demonstrated a preponderance of Man plus significant amounts of other sugars, even though subsequent analysis of the covalently bound sugars implicated Man only (10). To solve this dichotomy and to establish the nature of the full array of bound sugars, *M. tuberculosis* was labeled with [U-^{14}C]glucose under conditions that allowed for uniform labeling of all somatic sugars (9). The radiolabeled 45-kDa glycoprotein was exhaustively purified and subjected to acid hydrolysis. Analysis of the hydrolyzed sugars by high-performance anion-exchange chromatography demonstrated the presence of 3.19 nmol of mannose, 0.98 nmol of arabinose, 0.11 nmol of glucose, and a trace amount of galactose (Fig. 1B). However, only the Man component was radiolabeled (Fig. 1B), demonstrating that the other sugars were not of mycobacterial origin and probably arose from the various chromatographic supports used in the extensive purification steps applied to the 45-kDa glycoprotein.

Results of proteolysis with subtilisin. Previously, microcapillary liquid chromatography-MS and *m/z* 162 neutral loss scanning were used to identify four glycopeptides (S_1, S_3, S_6, and S_{11}) generated from a subtilisin digest of the 45-kDa glycoprotein (10). However, the conditions used to resolve these products allowed the S_{11} peptide only to be obtained in a form pure enough for structural analyses. Under present conditions, a well resolved HPLC map of the peptides from a subtilisin digestion of the 45-kDa protein was obtained (Fig. 2A). The

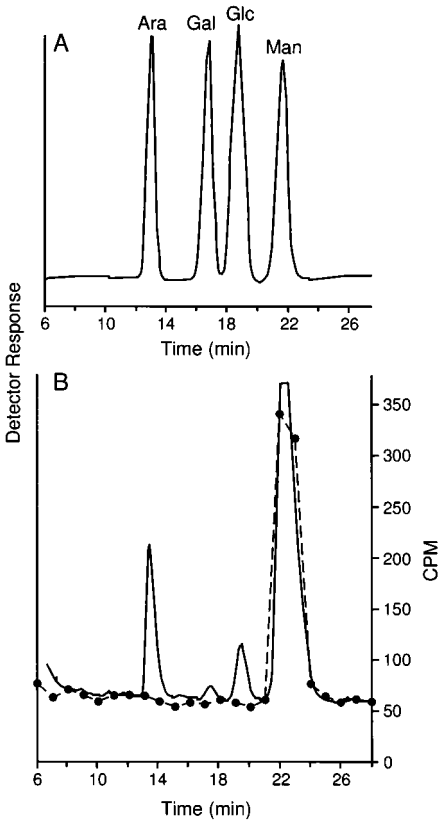

FIG. 1. Analysis of the sugar composition of the 45-kDa/MPT 32 glycoprotein. (A) Mixture of standard neutral sugar; (B) both unlabeled and ^{14}C-labeled sugars associated with 100 μg of the purified radiolabeled 45-kDa glycoprotein. Sugars were released by acid hydrolysis, resolved by high-performance anion-exchange chromatography, and detected with a pulsed amperometric detector (solid line) or by scintillation counting of fractions. The ^{14}C-labeled sugars are indicated by a dashed line. Ara, arabinose; Gal, galactose; Glc, glucose.

products were then subjected to microcapillary chromatography-MS and neutral loss scanning, seeking peptides that fragmented to give a daughter ion of *m/z* 162 or 132, indicative of the loss of a hexosyl or pentosyl unit(s), respectively. The digest yielded 50 peptides (S_2 to S_{51}), and of these, only S_7, S_{18}, S_{22}, S_{29}, and S_{41} produced daughter ions of *m/z* 162. No peptides that produced daughter ions of *m/z* 132 were detected (Fig. 2A), results which were in agreement with the compositional analysis that indicated only mannosylation of the 45-kDa glycoprotein.

N-terminal amino acid sequence and masses of the individual peptides were established by automated Edman degradation and FAB-MS (Table 1). The $(M+H)^+$ pseudomolecular ion of the S_{41} glycopeptide was observed at *m/z* 1515.5. Thus, this product was identical to the S_{11} glycopeptide described previously as the first bona fide glycopeptide in *M. tuberculosis* (10) with the structure DPEPAPPVPTTA-Man-Man. N-terminal sequencing confirmed this identity (Table 1). The $(M+H)^+$ pseudomolecular ion of the S_{29} glycopeptide was established as *m/z* 1150.5, and the corresponding amino acid sequence was established as XPVAPPPPAAA. The amino acid sequence of the S_{18} glycopeptide (*m/z* 1008.6) was identical to that of the S_{29} glycopeptide except for the absence of two Ala residues at the carboxyl terminus. Moreover, the difference between the $(M+H)^+$ pseudomolecular ions of the S_{29} and S_{18} peptides (*m/z* 142) corresponded to two Ala residues. N-terminal amino acid analysis of the S_{22} glycopeptide established the sequence GEVAPTPTXPTPQ. However, three individual $(M+H)^+$ pseudomolecular ions of *m/z* 1781.9, 1619.8, and 1457.6 were observed by FAB-MS for this S_{22} glycopeptide. The difference between these three ions was *m/z* 162, a result indicating differing levels of glycosylation of the same peptide. The S_{33} peptide produced an N-terminal sequence of GEV APTPTTPTPQ and an $(M+H)^+$ pseudomolecular ion of *m/z* 1295.5, indicating that it was the nonglycosylated version of S_{22} and that the site of glycosylation was the third Thr residue, the one that was not present in the *N*-terminal sequence of the S_{22} glycopeptide. The final peptide demonstrating a neutral loss of *m/z* 162 was the S_7 peptide, which exhibited an $(M+H)^+$ pseudomolecular ion of 954.4 and yielded the N-terminal sequence of ASPPSXA. For each of the glycopeptides, the difference in the observed mass and predicted mass was a factor of 162 when Thr was substituted for the unidentified amino acid (Table 1), a substitution confirmed by alignment with the deduced amino acid sequence of the 45-kDa protein (27). In addition, several other nonglycosylated peptides from this digestion were identified by FAB-MS, and the sequences of several of these were established by automated Edman degradation (Table 1).

Results of proteolysis with chymotrypsin-trypsin. Digestion

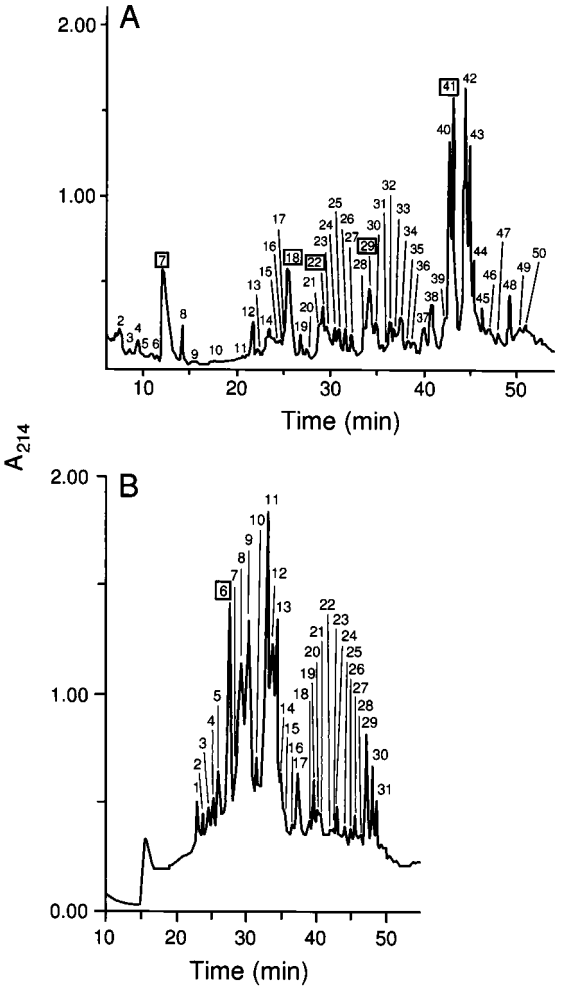

FIG. 2. Resolution and analysis of the peptides and glycopeptides of the 45-kDa glycoprotein digested with subtilisin (A) or digested with chymotrypsin-trypsin (B). Glycopeptides and peptides were resolved by reverse-phase HPLC. The glycopeptides were distinguished by the electrospray-MS neutral loss fragments of 162 AMU (boxes).

TABLE 1. FAB-MS and N-terminal amino acid sequence analysis of peptides generated by subtilisin digestion of 45-kDa glycoprotein from *M. tuberculosis*

Fragment	Position	Amino acid sequence[a]	Predicted mass[b]	Observed mass
S_7	13–19	**ASPPSXA**	630.3[c]	954.4
S_8	229–238	NNPVDKGAAK	1013.5	1013.5
S_{10}	123–128	**DTRIVL**	716.7	716.6
S_{12}	20–26	**AAPPAPA**	594.3	594.2
S_{13}	88–96	GWVESDAAH	971.4	972.0
S_{18}	27–35	**XPVAPPPPA**	846.6[c]	1008.6
S_{20}	142–152	**TDSKAAARLGS**	1076.6	1076.6
S_{21}	100–115	GSALLAKTTGDPPFPG	1528.8	1528.6
S_{22}	269–281	**GEVAPTPTXPTPQ**	1295.6[c]	1781.9
S_{22}	269–281	**GEVAPTPTXPTPQ**	1295.6[c]	1619.8
S_{22}	269–281	**GEVAPTPTXPTPQ**	1295.6[c]	1457.6
S_{25}	85–89	**LPAGW**	543.3	543.2
S_{26}	126–130	IVLGR	557.4	557.3
S_{29}	27–37	**XPVAPPPPAAA**	988.6[c]	1150.5
S_{30}	184–188	YYEVK	701.4	701.3
S_{33}	269–281	**GEVAPTPTTPTPQ**	1295.6	1295.5
S_{36}	36–46	**AANTPNAQPGD**	1055.6	1055.7
S_{37}	173–185	LDANGVSGSASYY	1303.6	1303.5
S_{38}	186–198	EVKFSDPSKPNGQ	1432.7	1432.7
S_{41}	1–12	**DPEPAPPVPXTA**	1191.6[c]	1515.5
S_{42}	201–208	TGVIGSPA	701.4	701.4
S_{43}	221–236	FVVWLGTANNPVDKGA	1686.9	1686.7
S_{44}	129–141	GRLDQKLYASAEA	1421.7	1421.9
S_{45}	158–165	**YMPYPGTR**	984.4	984.5
S_{46}	56–65	**PNAPPPPVIA**	972.6	972.6
S_{47}	168–173	QETVSL	676.4	676.4
S_{48}	203–220	**VIGSPAANAPDAGPPQRW**	1803.9	1804.1
S_{49}	79–84	**GGFSFA**	585.3	585.4
S_{50}	241–247	**AESIRPL**	785.5	785.5

[a] Amino acid sequences obtained by automated Edman degradation are shown in boldface type. Amino acid sequences inferred from FAB-MS analysis and alignment with the deduced amino acid sequence of the 45-kDa protein are shown in normal type.
[b] Monoisotopic mass of the predicted $(M+H)^+$ molecular ion.
[c] The predicted mass was calculated with Thr in the position of the unidentified amino acid.

of the 45-kDa glycoprotein with a mixture of chymotrypsin and trypsin produced a characteristic peptide map consisting of 31 individual peaks (Fig. 2B). Neutral loss scanning (*m/z* 162) revealed a single glycopeptide, CT_6. However, FAB-MS analysis of this glycopeptide yielded two $(M+H)^+$ pseudomolecular ions of *m/z* 3614.9 and 3453.6 (Table 2). The difference between these two was *m/z* 162, indicating the same peptide with various levels of glycosylation, like that observed for the S_{22} peptide cluster. The CT_6 peptide was rechromatographed by reversed-phase HPLC, resulting in resolution of the two peptides. FAB-MS analysis coupled with N-terminal amino acid sequencing demonstrated identical sequences of VAPP PAPAPAPAEPAPAPAPAGEVAPTPTTPTPQR but with a mono-Man unit on the *m/z* 3453.6 peptide, whereas the *m/z* 3614.9 peptide was diglycosylated. Thus, CT_6 represented a larger version of the S_{22} glycopeptide cluster. Additionally, as with the S_{22} cluster, a naturally nonglycosylated form of CT_6 was detected, CT_7 (*m/z* 3291.2) (Table 2). As with the peptides from the subtilisin digest, several nonglycosylated peptides from the chymotrypsin-trypsin digest were identified by FAB-MS and N-terminal amino acid sequencing (Table 2).

Detailed analyses of individual glycopeptides. A common characteristic of O-glycosylated proteins or peptides is the inability to clearly identify amino acids at sites of glycosylation by means of N-terminal sequencing (1). This problem was noted in the case of the glycopeptides generated by subtilisin digestion of the 45-kDa glycoprotein. Thus, the unidentifiable amino acid in each glycopeptide (Table 1) represented the site of glycosylation. The original analysis of the S_{41} peptide by secondary-ion MS demonstrated that this peptide was O glycosylated at a Thr residue (10). To confirm this earlier result and to determine whether the other glycopeptides were glycosylated in a similar manner, individual glycopeptides obtained from the subtilisin-digested material were analyzed by FAB-MS before and after digestion with α-mannosidase. Additionally, the oligoglycosyl alditols released from the glycopeptides by β-elimination were analyzed by GC-MS to identify sugar residues and their linkages.

S_7 glycopeptide. Initial FAB-MS of the α-mannosidase-digested S_7 peptide did not yield a detectable pseudomolecular ion signal. However, a strong signal was observed at *m/z* 708.2 when the same sample was N acetylated and methylesterified (Fig. 3B). The *m/z* difference between the undigested and core S_7 peptides indicated glycosylation with two α-Man residues (Fig. 3A). Additionally, the *m/z* values of the pseudomolecular ions of the glycosylated and deglycosylated–N-acetylated–methylesterified S_7 peptides were consistent with Thr being the unknown amino acid at position 6. Methylation analysis of the oligoglycosyl alditol released from the peptide by β-elimination demonstrated the presence of prereduced 2-linked and terminal Man residues (Table 3). Thus, the S_7 peptide possessed the amino acid sequence ASPPSTA and was O glycosylated at the Thr residue with α1-2-linked mannobiose (Fig. 3).

S_{22} glycopeptide. As stated previously, the broad peak corresponding to the S_{22} peptide consisted of three individual peptides, all of which contained the same amino acid sequence but differed in the extent of their glycosylation. This cluster of peptides was rechromatographed by reversed-phase HPLC,

TABLE 2. FAB-MS and N-terminal amino acid sequence analysis of peptides generated by chymotrypsin-trypsin digestion of the 45-kDa protein from *M. tuberculosis*

Fragment	Position	N-terminal amino acid sequence analysis[a]	Predicted mass[b]	Observed mass
CT_4[c]	176–184	NGVSGSASY	841.8	842.3
	126–130	IVLGR	557.4	557.7
CT_5	201–219	**TGVIGSPAANAPDAGPPQR**	1775.9	1776.7
CT_6[c]	248–282	**VAPPPAPAPAPAEPAPAPAPA**GEV		
		APTPTTPTPQR	3290.4	3614.9
		VAPPPAPAPAPAEPAPAPAPAGEV		
		APTPTTPTPQR	3290.4	3453.6
CT_7	248–282	**VAPPPAPAPAPAEPAPAPAPAGE**V		
		APTPTTPTPQR	3290.4	3291.2
CT_8[c]	107–125	**TTGDPPFPGQPPPV**ANDTR	1964.0	1964.9
	137–149	ASAEATDSKAAAR	1248.6	1248.2
CT_9[c]	126–134	IVLGRLDQK	1041.6	1042.1
	74–81	**IDNPVGGF**	818.4	818.6
CT_{10}	201–220	**TGVIGSPAANAPDAGPPQRW**	1962.0	1962.6
CT_{12}	186–200	EVKFSDPSKPNGQIW	1731.9	1733.0
CT_{17}[c]	225–247	**LGTANNPVDKGAAKALAESIRPL**	2307.4	2307.4
	90–99	VESDAAHFDY	1153.5	1153.5
CT_{19}	82–89	**SFALPAGW**	848.4	848.3
CT_{25}	84–106	**ALPAGWVESDAAHFDYGSALLAK**	2390.2	2391.4
CT_{27}	169–175	**ETVSLDA**	734.9	735.3

[a] Amino acid sequences obtained by automated Edman degradation are shown in boldface type. Amino acid sequences inferred from FAB-MS analysis and alignment with the deduced amino acid sequence of the 45-kDa glycoprotein are shown in normal type.
[b] Monoisotopic mass of the predicted $(M+H)^+$ molecular ion.
[c] Two $(M+H)^+$ molecular ions were observed by FAB-MS.

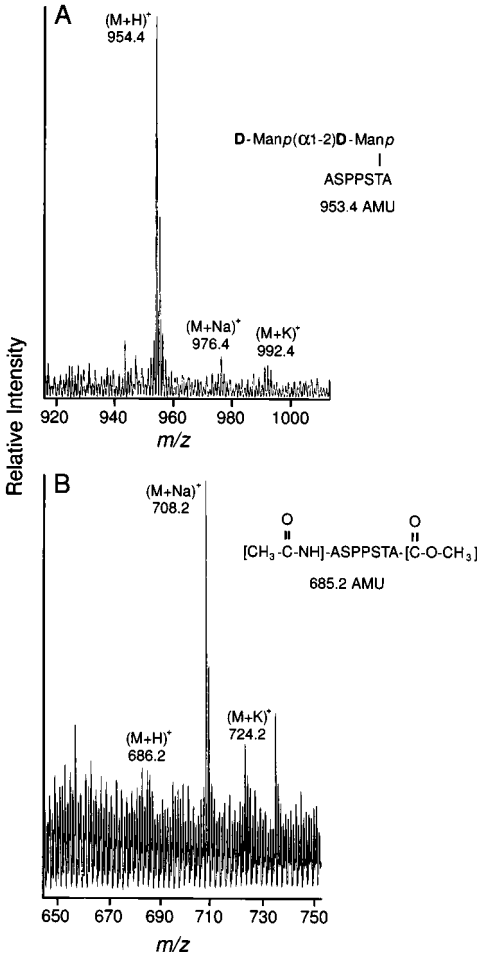

FIG. 3. FAB-MS analysis of the S_7 peptide. (A) Fully glycosylated S_7 peptide; (B) α-mannosidase-digested S_7 peptide. The amino acid sequence of this peptide was obtained by N-terminal amino acid sequencing. The extent of glycosylation and the identity of the glycosylated amino acid were inferred from the mass difference between the molecular ions afforded by α-mannosidase-digested, methylesterified, N-acetylated S_7 peptide (B) and the fully glycosylated form (A). The linkage of the mannose residues was elucidated by GC-MS methylation analysis of the oligoglycosyl alditol released by β-elimination.

and the largest of the three peaks was selected for further FAB-MS analysis. The *m/z* difference between the $(M+H)^+$ pseudomolecular ions of the glycosylated and deglycosylated forms of the S_{22} peptide was *m/z* 324 (Fig. 4), demonstrating that this component peptide of the S_{22} cluster was glycosylated with two α-Man residues. N-terminal sequencing of the α-mannosidase-digested form of this S_{22} peptide produced a sequence identical to that obtained for the naturally occurring nonglycosylated S_{33} peptide (GEVAPTPTTPTPQ), confirming that the S_{22} glycopeptide was O glycosylated at the third Thr residue. Similar analyses of the two other glycopeptides of the S_{22} cluster confirmed that one (*m/z* 1781.9) was glycosylated with three α-Man residues and the other (*m/z* 1457.6) was glycosylated with a single α-Man residue. Analysis of the (oligo)glycosyl alditols further demonstrated heterogeneous glycosylation of the peptide with α-Man, (α1-2)-mannobiose, or (α1-2)-mannotriose.

S_{29} glycopeptide. FAB-MS analysis of the α-mannosidase-treated S_{29} glycopeptide yielded two $(M+H)^+$ pseudomolecular ions of *m/z* 1150.5 and 988.5, indicating only partial removal of the α-Man residues (Fig. 5). Nevertheless, the 988.5 $(M+H)^+$ pseudomolecular ion corresponded to the fully deglycosylated form of this peptide when Thr was substituted for the unknown N-terminal amino acid. Sugar analysis of the S_{29} glycopeptide further demonstrated glycosylation by a single Man residue (data not shown). Together these results demonstrated that the N-terminal Thr of this peptide (TPVAPPP PAAA) was glycosylated with a single α-Man residue (Fig. 5). As expected, similar analyses of S_{18} gave identical results for the nature and location of glycosylation (data not shown).

S_{41} glycopeptide. Previously, the S_{41} glycopeptide was shown to be O glycosylated at the position 10 Thr residue (10). α-Mannosidase digestion followed by N acetylation, methylesterification, and FAB-MS analysis produced an $(M+H)^+$ pseudomolecular ion of *m/z* 1297.6 (Fig. 6B), indicating the loss of two α-Man residues. The oligoglycosyl alditol released from the S_{41} glycopeptide was found to consist of a prereduced 2-linked mannitol and a terminal Man (Table 3). Thus, the Thr residue at position 10 of this peptide was glycosylated with an (α1-2)-linked mannobiose (Fig. 6).

In all, these results demonstrated that there were four sites of glycosylation on the 45-kDa MPT 32 glycoprotein and that each site of glycosylation consisted of a Thr residue O-linked to an α-mannose, α-mannobiose, or α-mannotriose. All of the glycosidic linkages were determined to be α1-2. Of the four glycosylation sites, only one appeared to possess heterogeneity in the number of mannosyl residues present.

DISCUSSION

Both in this present and previous work (10), the application of proteolysis, full resolution of both peptides and glycopeptides by reversed-phase HPLC, and MS analysis coupled with neutral loss scanning were essential in defining the sites of protein glycosylation and the nature and length of the glycosyl substituents. However, the alignment of these glycopeptides within the mature polypeptide backbone and the advancement

TABLE 3. GC-MS analysis of partially methylated alditol acetates from the permethylated oligoglycosyl alditols released by β-elimination of the glycopeptides obtained by subtilisin digestion of the 45-kDa glycoprotein

Peptide	Retention time (min)[a]	Fragment ions of the partially methylated alditol acetates (*m/z*)	Sugar[b]
S_7	9.49	129, 145, 161, 205	Prereduced, 2-linked Man
	11.02	102, 118, 129, 145, 161, 162, 205	Terminal Man
S_{22}[c] [$(M+H)^+$ *m/z* 1619.8]	9.49	129, 145, 161, 205	Prereduced, 2-linked Man
	11.02	102, 118, 129, 145, 161, 162, 205	Terminal Man
S_{22}[c] [$(M+H)^+$ *m/z* 1781.8]	9.50	129, 145, 161, 205	Prereduced, 2-linked Man
	11.03	102, 118, 129, 145, 161, 162, 205	Terminal Man
	12.03	129, 130, 161, 190	2-Linked Man
S_{41}	9.50	129, 145, 161, 205	Prereduced, 2-linked Man
	11.03	102, 118, 129, 145, 161, 162, 205	Terminal Man

[a] Retention time of the partially methylated alditol acetates separated by GC.
[b] The sugar residues were identified based on the diagnostic ions observed in the mass spectrum of the partially methylated alditol acetates and by referring to the retention time of authentic standards.
[c] The S_{22} glycoside was rechromatographed by reversed-phase HPLC to obtain the individual glycopeptides.

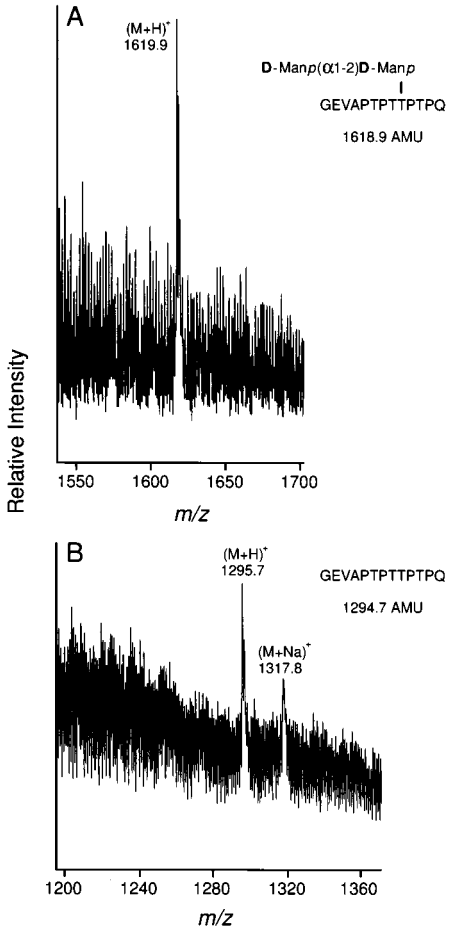

FIG. 4. FAB-MS analysis of the dominant peptide of the S_{22} peptide cluster. (A) Diglycosylated S_{22} peptide; (B) α-mannosidase-digested S_{22} peptide. The broad experimental approach is described in the legend to Fig. 3.

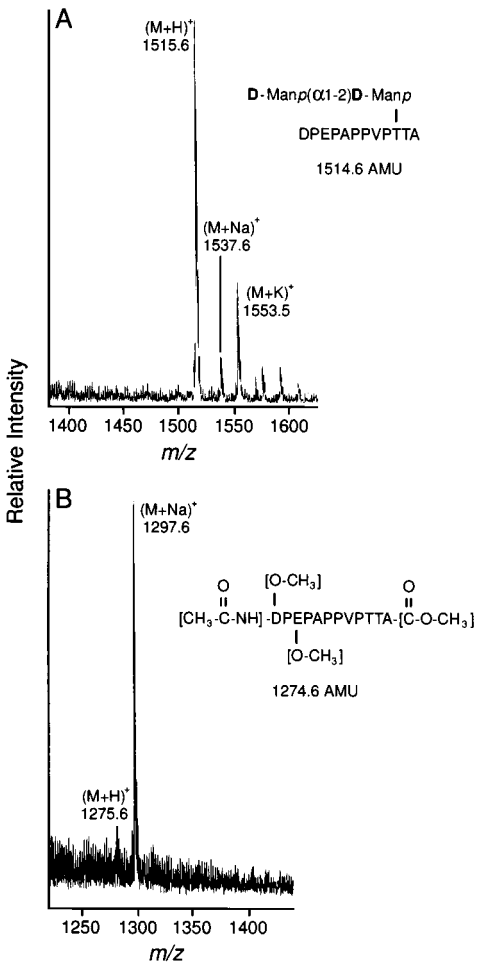

FIG. 6. FAB-MS analysis of the S_{41} peptide. (A) Fully glycosylated S_{41} peptide; (B) α-mannosidase-digested, methylesterified, N-acetylated S_{41} peptide. Further experimental detail is described in the legend to Fig. 3.

of a complete structure for the 45-kDa glycoprotein (Fig. 7) were possible only with the recent elucidation of the gene sequence of this protein (27). In sum, the combined approaches allowed us to establish that four glycosylation sites lie within Pro-rich domains of the N-terminal and C-terminal regions of the mature 45-kDa glycoprotein (Fig. 7). Specifically, Thr residues at amino acid positions 10 and 18 are O glycosylated with the mannobiose unit α-D-Man*p*(1→2)-α-D-Man*p*, the Thr-27 is substituted with a single α-D-Man*p* unit, while Thr-277 (in the C-terminal region) may be linked to a α-D-Man*p*, α-D-Man*p*(1→2)-D-Man*p*, or α-D-Man*p*(1→2)-α-D-Man*p*(1→2)-D-Man*p* unit (Fig. 7). Such glycosylation microheterogeneity is consistent with other well described prokaryotic glycoproteins (28, 44).

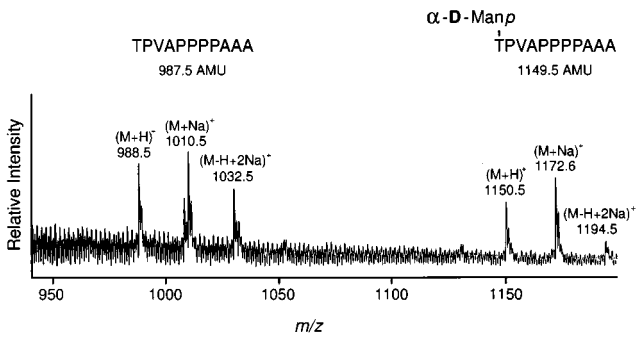

FIG. 5. FAB-MS analysis of the α-mannosidase-digested S_{29} peptide. See the legend to Fig. 3 for further information.

The presence of glycoproteins in both archaebacteria and eubacteria is well established. Additionally, N- and O-linked glycosyl units, including the unique O glycosylation of Tyr residues, are now recognized (3, 15, 28, 31, 32, 44). Of these, only the P40 protease of *Flavobacterium meningosepticum* is known to possess Man residues O-linked to a Ser or Thr, which interestingly are in the form of a mannooligosaccharide rather than single Man*p* units or a mannobiose or mannotriose (36, 37). The other known O-glycosylated bacterial proteins contain a GlcNAc unit O-linked to either Thr or Ser (44). In fact, the nature of O glycosylation of the 45-kDa protein of *M. tuberculosis* is more reminiscent of the simpler sites of O glycosylation found in the yeast mannoproteins (29).

A comparison of glycosylated proteins confirms that a strict consensus sequence is not required for O glycosylation (18). Nevertheless, several loose amino acid motifs for O glycosylation are obvious in this and previous work (17, 18). The most commonly observed site is a Ser or Thr residue within Pro-rich domains (17). More specifically, it appears that the O glycosy-

FIG. 7. Alignment of the glycopeptides and peptides generated by subtilisin and chymotrypsin-trypsin digestion of the 45-kDa glycoprotein with the deduced amino acid sequence of the mature protein. The dashed line indicates the N-terminal amino acid sequence obtained from the intact 45-kDa glycoprotein, and the solid lines indicate the location of individual glycopeptides or peptides. The boxed threonine residues indicate the sites of glycosylation.

lation of a Ser or Thr is increased significantly when a Pro residue is located at positions −1 or +3 relative to the glycosylated amino acid (17). Originally, we proposed that this motif applied in the case of the mycobacterial 45-kDa glycoprotein (10). However, in this current study, the sequence data on individual glycopeptides illustrated a motif possessing at least two Pro residues located within a four-amino-acid stretch upstream of the glycosylated Thr. Several earlier reports indicate that efficient O glycosylation of hydroxyamino acids is dependent on the proper conformation of the glycosylated area as well as the specific amino acid motif (5). It is possible that the proposed O-glycosylation motif is not a strict motif per se. Rather, the Pro residues within the proximity of the O-glycosylation sites serve more to expose specific Thr residues to allow for their ready recognition by glycosyltransferases. This hypothesis may explain why Ser-5 of S_7, Thr-11 of S_{41}, and Thr-6, -9, and -11 of S_{22} are not glycosylated, even though they fit the proposed structural definition of an O-glycosylation motif (17).

Evaluations of the effects of O glycosylation on the conformation of proteins indicate that the presence of sugar units in heavily clustered domains limits protein folding, leading to extended conformations of the protein in these areas (16, 24). Thus, in the context of the 45-kDa glycoprotein, it is probable that the combination of glycosyl units in the vicinity of a preponderance of Pro residues will ensure a minimum of secondary or tertiary structure leading to a "stiff," extended conformation. Such a feature would explain the dramatic difference between the predicted molecular weight of 45 kDa based on mobility in SDS-PAGE and the true molecular weight of 30.2 kDa (27).

It is tempting to speculate that glycosylation plays a role in the transport of the 45-kDa glycoprotein across the cellular envelope of *M. tuberculosis* analogous to the enhanced secretion observed for the O-glycosylated form of the cellulase produced by *Trichoderma reesei* (25). The gene sequence encoding the 45-kDa glycoprotein demonstrates the presence of a signal peptide (27) similar to that of many other *M. tuberculosis* culture filtrate proteins (50). However, in contrast to the 45-kDa glycoprotein, a large number of other *M. tuberculosis* culture filtrate proteins are associated with the cell wall (2). Accordingly, it is possible that the extended conformation predicted for the N- and C-terminal regions of the 45-kDa protein combined with extensive glycosylation in these regions is responsible for the exclusive targeting of this protein to the extracellular environment. Indeed, Laqueyrerie et al. (27) had suggested that this 45-kDa protein may contribute to the uptake and transport of metabolites across the mycobacterial cell wall and cytoplasmic membrane, which themselves are endowed with unusual physicochemical attributes (4).

A perusal of the older literature provides clues as to the possible biological source of the Man substituents of the 45-kDa glycoprotein and the mechanism of mannosylation. Schultz and Takayama (41) had observed the mannosylation of an unknown *M. smegmatis* protein catalyzed by a particulate cell-free fraction of the organism, presumably cytoplasmic membranes. Moreover, the donor of the Man units was identified as one or both of two mannosylphosphorylpolyisoprenols, i.e., mannosyl-1-phosphoryl-octahydroheptaprenol and/or mannosyl-1-phosphoryl-decaprenol (47). Additionally, these earlier studies indicated that GDP-mannose acts as the mannosyl donor for the biosynthesis of the mannosylphosphorylpolyisoprenols and is not the direct donor of the peptide-linked mannoses (41). Accordingly, mannosylation of mycobacterial proteins

may bear similarities to that of the yeast mannoproteins (20). We are also conscious of the fact that the di- and tri-mannosyl units of the 45-kDa glycoprotein are identical to the mannose caps of mycobacterial lipoarabinomannan (the so-called Man-lipoarabinomannan) in absolute configuration and linkage (7), with the insinuation that the enzymatic machinery is shared by both systems; such a phenomenon is observed for the enzymes involved in the synthesis of yeast mannoproteins and mannans (20). Furthermore, the mannosyl units of the 45-kDa protein may share a role in the phagocytosis of *M. tuberculosis*, analogous to that of the Man-lipoarabinomannan (40).

Present and past work (10) has resulted in categorical proof of the existence of protein glycosylation in mycobacteria. The challenge now is to define the mechanism of glycosylation and its importance in the physiology of *M. tuberculosis* and its pathogenesis. The full definition of the gene encoding the mature 45-kDa MPT 32 glycoprotein (27), the utilization of site-directed mutagenesis, and the recent development of methods for the generation of knockout mutants in *M. tuberculosis* (35) ensure the resolution of these questions.

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