Identification of a Novel Mycobacterial Arabinosyltransferase Activity Which Adds an Arabinosyl Residue to α-D-Mannosyl Residues

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Supporting Information

ABSTRACT: The arabinosyltransferases responsible for the biosynthesis of the arabinan domains of two abundant heteropolysaccharides of the cell envelope of all mycobacterial species, lipoarabinomannan and arabinogalactan, are validated drug targets. Using a cell envelope preparation from Mycobacterium smegmatis as the enzyme source and di- and trimannoside synthetic acceptors, we uncovered a previously undetected arabinosyltransferase activity. Thin layer chromatography, GC/MS, and LC/MS/MS analyses of the major enzymatic product are consistent with the transfer of an arabinose residue to the 6 position of the terminal mannosyl residue at the nonreducing end of the acceptors. The newly identified enzymatic activity is resistant to ethambutol and could correspond to the priming arabinosyl transfer reaction that occurs during lipoarabinomannan biosynthesis.

Tuberculosis is the most deadly infectious disease in the world, killing 1.5 million people annually.1 The continuing rise of multidrug-resistant Mycobacterium tuberculosis places a high priority on the development of new chemotherapeutics with novel modes of action. In this context, elucidating the biosynthetic pathways allowing M. tuberculosis to synthesize and assemble its complex cell envelope represents a crucial area of research. Two essential D-arabinofuranosyl-containing heteropolysaccharides, arabinogalactan (AG) and lipoarabinomannan (LAM) (Figure 1), populate the cell envelope of all Mycobacterium species.2 These complex glycoconjugates play various critical roles in the physiology of mycobacteria and their interactions with the host.3 Owing to their central involvement in the elongation and branching of the arabinan domains of AG and LAM, arabinosyltransferases (AraTs) and the enzymes involved in the formation of decaprenyl-phosphoryl-β-D-arabinofuranose (DPA)—the arabinosyl donor used in all AraT-mediated transfer reactions—are essential enzymes whose therapeutic potential has been well validated.2,3 On the basis of the structural organization of the arabinan domain of LAM (Figure 1), at least four different linkage-specific AraTs are likely to be needed to complete its biosynthesis. To date, AftC, an α-(1→3) branching AraT,4 AftD, another branching AraT,5 and EmbC, a proposed α-(1→5) arabinan chain elongating enzyme,6–10 are the only AraT’s shown to be involved in the LAM pathway, while the priming AraT responsible for the transfer of the first arabinosyl residue onto the mannan backbone of LAM, and the capping AraT(s) responsible for the β-(1→2) linkages at the nonreducing end of the arabinan domain specific to LAM remain unknown. In the present study, a cell-free assay using synthetic mannoside acceptors and Mycobacterium smegmatis membrane and envelope preparations was developed to probe the priming AraT activity specific to LAM biosynthesis. We and others have previously evaluated the effectiveness of several synthetic neoglycolipids as substrates for mycobacterial glycosyltransferase activities.11,12 Specifically, di- and trimannosides with an octyl aglycon chain (Figure 2a) have shown acceptor capabilities with α-(1→6) mannosyltransferases in cell-free assay systems.13–15 Using synthetic galactosyl acceptors, earlier studies probed the priming AraT activity of the AG pathway.16,17 As part of our continuing research toward understanding LAM biosynthesis, we sought to address the question of the priming AraT activity of the LAM pathway.

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using a variety of synthetic mannoside acceptors (Figure 2a, 1–4) and a membrane plus cell envelope preparation from \textit{M. smegmatis} as the enzyme source. First, a radioactivity-based assay was developed to test the synthetic disaccharide Man-(1→6)-Man-(1→octyl (1) as an arabinose acceptor. DP[14C]A, generated \textit{in situ} from p[14C]Rpp, served as the arabinosyl donor (Figure 2a).\textsuperscript{5} Thin layer chromatography (TLC) analysis of the radiolabeled enzymatic products followed by autoradiography revealed the formation of a single radioactive compound migrating slower than the dimannoside acceptor that was absent from the control reaction lacking acceptor 1 (Figure 2b). Thus, \textit{M. smegmatis} extracts contain an enzyme with AraT activity capable of transferring [14C]arabinose onto acceptor 1.

The front-line antituberculosis drug, ethambutol (EMB), is known to inhibit the EmbA, EmbB and EmbC AraTs.\textsuperscript{18–20} The addition of high concentrations of EMB to the reaction mixture (100 and 200 \(\mu\)g mL\(^{-1}\); the MIC of EMB against the \textit{M. smegmatis} strain used in this study is 7.5 \(\mu\)g mL\(^{-1}\)), however, had no significant effect on the transfer of an arabinosyl residue to acceptor 1 (Figure 2c).

To facilitate the characterization of the major enzymatic product of the reaction, a similar AraT assay as described above was next performed using nonradioactive DPA synthesized in-house\textsuperscript{21} as the direct arabinofuranosyl donor (Figure 2a). 1-Butanol-extracted enzymatic products were per-O-acetylated as described in the Supporting Information Methods, and the products were analyzed by LC/MS with high mass resolution. Both the unreacted acceptor substrate 1 (marked with an arrow in Figure 3b) and the major enzymatic product (marked with an asterisk in Figure 3b) were detected by this method as peaks eluting around 27 min. These peaks were not present in the negative control reaction lacking the dimannoside acceptor 1 (Figure 3a). The unreacted acceptor 1 and its single arabinose residue-containing enzymatic product were identified by extracted ion chromatograms (EICs) of their corresponding [M+NH\(_4\)]\(^+\) ions (Figure 3c–f); the relative amounts of the compounds can be noted by the values of the y-axis. The mass spectrum of the unreacted dimannoside acceptor 1 revealed strong pseudomolecular ions with \(m/z\) 766.35 and 771.30 as ammonium and sodiated adducts (Figure 3g). Most notable was the presence of mass spectrum ions \(m/z\) 982.41 [M+NH\(_4\)]\(^+\) and 987.36 [M + Na]\(^+\) for the enzymatic product (Figure 3h). The presence of these ions confirms that a single arabinosyl residue was enzymatically transferred to give the product Ara\(_{f}-(1\rightarrow 3)-\text{Man}-(1 \rightarrow 6)-\text{Man}-(1 \rightarrow \text{octyl})\). Interestingly, we also identified small amounts of an arabinose-containing tetrasaccharide enzymatic product at \(m/z\) 1270.49 [M + NH\(_4\)]\(^+\) (Figure 3j), suggesting that the Man-(1 \→ 6)-Man-(1 \→ octyl) acceptor was endogenously converted to the...
trimannoside Manp-(1 → 6)-Man-(1 → 6)-Man-(1 → octyl) (as shown on the EIC in Figure 3e), which then served as an acceptor in the AraT reaction. The presence of Manp-(1 → 6)-Man-(1 → 6)-Man-(1 → octyl) was confirmed by the presence of m/z 1054.43 [M+NH4]+ as shown in Figure 3i. Presumably, mannosylation of the acceptor occurred due to the presence of an endogenous mannosyltransferase and mannose donor in the reaction mixture.

Synthetic mannoside acceptors of varying chain lengths were next tested in the AraT assay to compare their effectiveness as acceptor substrates (see Figure 2a, acceptors 1–3). We found that the AraT activity was predominant with dimannoside (1) compared to the trimannoside (2; Figure 2c), and we were not able to detect any transfer activity with the pentamannoside substrate (3; data not shown). Stability issues with the pentamannoside acceptor may account for the latter result as
we found this substrate to be degraded into di-, tri-, and tetramannosides, consistent with earlier observations.14

To determine the identity of the mannosyl residue modified by the arabinose on the Man-(1 → 6)-Man-(1 → octyl acceptor, and the nature of its linkage, the enzymatic products were per-O-methylated22 and analyzed by LC/MS/MS. O-methyl derivatives of major (peak I) and minor (peak II) Ara-Man₂-octyl products were detected. The major per-O-

Figure 3. LC/MS analysis of the AraT reaction products. Membrane and cell envelope preparations from M. smegmatis were incubated without arabinosyl donor or mannoside acceptor, or with DPA donor and acceptor I. Traces a and b show the total ion chromatograms of the control lacking substrates and the complete reaction, respectively. A series of peaks centered around 27 min is present in the substrate containing a reaction (b) but not in the control (a). Unreacted substrate I was identified using selected ion monitoring of the [M+NH₄]⁺ ion at m/z 766.34 (c); the mass spectrum is shown in g. The monoarabinosylated product was identified using selected ion monitoring of the [M+NH₄]⁺ ion at m/z 982.41; the mass spectrum is shown in h. Even though no mannoside donors (GDP-Man or polyprenyl-phosphomannose) were added to the reaction, the presence of endogenous mannose donor in the membrane fraction allowed for the monomannosylation of acceptor 1 as shown by selected ion monitoring of the [M+NH₄]⁺ ion at m/z 1054.43. This trimannoside was also arabinosylated as shown by selected ion monitoring of the [M+NH₄]⁺ ion at m/z 1270.49; the mass spectrum is shown in j. In all mass spectra, the ion at 5 amu higher mass than the [M + NH₄]⁺ ion corresponds to the [M + Na]⁺ ion.
methylated enzymatic product (peak I; Figure 4a), with a precursor ion at \(m/z\) 735.42 \([M + Na]^+\) was selected and fragmented by high energy (60 V) CID MS/MS (Figure 4b). The MS/MS fragmentation pattern was consistent with a linear enzymatic product rather than a branched one (Figure 4b). Thus, fragment ions at \(m/z\)‘s of 419.17 (C2), 357.22 (Y1), 401.17 (B2) and 461.19 (O,4A3) can only be formed with these \(m/z\) values from a linear trisaccharide. The lack of ions consistent with a nonreducing terminal mannosyl residue confirms the linear structure.

The internal cross ring cleavage of the internal mannosyl residue allowed the position on the mannosyl residue to be postulated in the same MS/MS analysis. Surprisingly, the results were consistent with the position of the arabinosyl substitution being on O-6 of the mannosyl residue and not on O-2 as expected from earlier data from our laboratories,23 which postulated that the arabinan was attached to O-2 of a mannosyl residue. This is shown by the three ions at \(m/z\) 257.13 (\(^{0,4A}_3\)A), 285.11 (\(^{3A}_3\)A) and 301.14 (\(^{0,3A}_3\)A) (Figure 4b). The \(^{0,3A}_3\)A and \(^{0,4A}_3\)A ions are consistent with the arabinosyl residue on O-4 or O-6; the \(^{0,4A}_3\)A ion is consistent only with the arabinosyl residue being present on O-6 of the mannosyl residue. Although somewhat weak, an E ion at \(m/z\) 371.17 is not consistent with the arabinosyl residue being attached at O-2. We thus conclude from our LC/MS/MS analysis that the arabinosyl residue is attached to O-6 of the terminal mannosyl residue located at the nonreducing end of acceptor 1 and has the structure Ara\(\beta-(1\rightarrow6)\)-Man\(\beta-(1\rightarrow6)\)-Manp-(1\rightarrow octyl.

To confirm the structure of the major product (peak I, Figure 4a), it was partially purified by LC using two columns in tandem. The partially purified product was then hydrolyzed, reduced with NaBD\(_4\), and acetylated, and the resultant partially methylated partially acetylated alditols were analyzed by GC/MS. Unfortunately, the major Ara-Man\(_2\)-octyl product copurified with substantial amounts of Man\(_2\)-octyl substrate resulting in considerable amounts of the partially methylated partially acetylated alditols from the substrate. Also, the amounts of the desired compounds were low compared to contaminants. However, by use of selected ion chromatographs, it was clearly shown that the expected 2,3,5-tri-O-methyl-1,4-di-
The present study is the first report of an EMB-resistant mycobacterial (1-O-acetyl arabinitol (t-Araf)) from the product, 2,3,4-tri-O-methyl-1,5,6-tri-O-acetyl mannosil (6-Manp) from both substrate and product, and 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl mannosil (t-Manp) from the substrate were present (see Supporting Information Figure 1). In addition, 3,4,6-tri-O-methyl-1,2,5-tri-O-acetyl mannosil, 2,4,6 tri-O-methyl-1,3,5-tri-O-acetyl mannosil, and 2,3,6-tri-O-methyl-1,4,5-tri-O-acetyl mannosil products expected from 2-, 3-, and 4-linked mannosne were not present as shown using selected ion chromatographs of ions specific for these compounds.

The second quantitatively minor Ara-Man-ocetyl (peak II; Figure 4a) was also analyzed by LC/MS/MS. Due to its low amounts, we were unable to obtain complete structural information on it. The MS/MS spectrum was consistent, for the most part, with the arabinosyl residue being attached to the interior mannosyl residues as shown in (see Supporting Information Figure 2). Thus, it is possible that this molecule is the expected Ara-(1→2)-[Man-(1→6)]-Man-(1→octyl, but the spectrum certainly does not show the linkages clearly and even shows some ambiguity in regards to the branched nature of the component.

Finally, to assess the dependence of the new AraT activity detected herein for the O-6' hydroxyl group of the terminal mannosyl residue of acceptor, nonradiolabeled assays were repeated using C6-deoxygenated disaccharide as an acceptor substrate (Figure 2a, acceptor 4). This substrate was previously reported to serve as an acceptor for unknown mannosyltransferase. As expected, we did not observe any (1→6) AraT activity with this substrate (Figure 2c).

The present study is the first report of an EMB-resistant mycobacterial (1→6) AraT capable of utilizing synthetic (1→6)-Manp disaccharide and trisaccharide as acceptors in the formation of arabinosyl-containing tri- and tetra-saccharides. The structure of the major product using the dimannoside acceptor as Ara-(1→2)-[Man-(1→6)]-Man-(1→octyl) (as determined by GC/MS and further supported by GC/MS results) was surprising given an earlier study by Chatterjee et al., which had proposed that the priming arabinose on the mannabone of LAM from M. tuberculosis was on the 2 position of a 2,6-linked mannosyl residue. Thus, as frequently is the case, biosynthetic data supplements pure structural data, and to investigate the reasons for this discrepancy, studies are being undertaken in our laboratory to revisit the linkage of the priming arabinosyl residue in the LAM of M. smegmatis and M. tuberculosis. Preliminary studies based on the enzymatic degradation of the truncated LAM produced by an embC knockout mutant of M. smegmatis indicate that, at least in this species, significant amounts of an arabinosyl residue attached at O-6 of a mannosyl residue are present (unpublished work in progress), consistent with the major enzymatic activity detected herein. Whether M. tuberculosis LAM and M. smegmatis LAM are different in this respect is still under investigation. The presence of a minor product which might be an arabinosyl residue attached to the 2 position of a 6-linked mannosyl product (although this structure is far from demonstrated) further complicates our understanding of LAM structure and its biosynthesis. It is possible that arabinosyl residues are found attached to mannosyl residues in two ways, and thus, we emphasize that the finding of the present work requires detailed structural investigation of LAM from both M. smegmatis and M. tuberculosis.

Another important aspect of the present work is the methodology developed to identify the priming arabinosyl-transferase(s) of LAM. Overexpression of candidate genes in M. smegmatis followed by enzymatic analysis as described herein should result in an increase in the amounts of the major or minor products depending on enzymatic activity. Such overexpression studies, along with the above-mentioned structural studies, are in progress in our laboratory.

**METHODS**

**Arabinosyltransferase Assay.** Enzymatically active membranes and cell envelope (P60) fractions from M. smegmatis mc²155 were prepared as described previously. The [14C]-labeled arabinoside donor, phosphoribosyl pyrophosphate (p[14C]Rpp), was generated from uniformly labeled D-[14C] glucose (American Radiochemical Inc.) as described. An AraT assay reaction mixture contained synthetic mannoside acceptors (see Figure 2a and Supporting Information Methods; 0.2 mM), ATP (1 mM), p[14C]Rpp (500 000 dpm), buffer A [50 mM MOPS (pH 8), 5 mM 2-mercaptoethanol, 10 mM MgCl₂, membrane (0.5 mg), and P60 (0.3 mg)] fractions in a total volume of 200 μL. Negative control reactions lacked acceptor substrates. Reaction mixtures were incubated at 37 °C for 2 h and terminated by the addition of 200 μL of ethanol. Upon centrifugation at 14 000 rpm for 10 min, the resulting supernatant was loaded onto a strong anion exchange column (Hypersep SAX; Thermo scientific) pre-equilibrated in water. The products were eluted from the column with 2 mL of 50% ethanol solution, and the eluents evaporated to dryness and finally partitioned between 1-butanol and water (1:1). Butanol fractions were recovered, and the lower aqueous phase was further extracted twice with 1-butanol. The pooled butanol fractions were backwashed with 1-butanol, dried, and resuspended in 200 μL of 1-butanol. Equal volumes of radiolabeled products were applied to aluminum-backed silica gel 60 F254 TLC plates and developed in CHCl₃/CH₃OH/13 M NH₄OH/1 M NH₄OAc/H₂O (180:140:9:9:23 by vol.) followed by autoradiography at ~80 °C using Biomax MR1 films (Kodak). Nonradioactive AraT assays were performed as above in the presence of DPA (100 μM) synthesized in-house.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00093. Supporting Methods section and supporting Figures 1 and 2 (PDF)

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


