Decaprenylphosphoryl Arabinofuranose, the Donor of the d-Arabinofuranosyl Residues of Mycobacterial Arabinan, Is Formed via a Two-Step Epimerization of Decaprenylphosphoryl Ribose

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The major cell wall polysaccharide of mycobacteria is a branched-chain arabinogalactan in which arabinan chains are attached to the 5 carbon of some of the 6-linked galactofuranose residues; these arabinan chains are composed exclusively of d-arabinofuranose (Ara) residues. The immediate precursor of the polymerized Ara is decaprenylphosphoryl-d-Ara, which is derived from 5-phosphoribose 1-diphosphate (pRpp) in an undefined manner. On the basis of time course, feedback, and chemical reduction experiment results we propose that decaprenylphosphoryl-Ara is synthesized by the following sequence of events. (i) pRpp is transferred to a decaprenyl-phosphate molecule to form decaprenylphosphoryl-β-o-5-phosphoribose. (ii) Decaprenylphosphoryl-β-o-5-phosphoribose is dephosphorylated to form decaprenylphosphoryl-β-o-ribose. (iii) The hydroxyl group at the 2 position of the ribose is oxidized and is likely to form decaprenylphosphoryl-2-keto-β-o-erythro-pentofuranose. (iv) Decaprenylphosphoryl-2-keto-β-o-erythro-pentofuranose is reduced to form decaprenylphosphoryl-β-o-Ara. Thus, the epimerization of the ribosyl to an arabinosyl residue occurs at the lipid-linked level; this is the first report of an epimerase that utilizes a lipid-linked sugar as a substrate. On the basis of similarity to proteins implicated in the arabinosylation of the Azorhizobium cauli dian nodulation factor, two genes were cloned from the Mycobacterium tuberculosis genome and expressed in a heterologous host, and the protein was purified. Together, these proteins (Rv3790 and Rv3791) are able to catalyze the epimerization, although neither protein individually is sufficient to support the activity.

The cell envelope of Mycobacterium tuberculosis is made up of three major components: a plasma membrane, a covalently linked mycolic acid, arabinogalactan, and peptidoglycan complex, and polysaccharide-rich capsule-like material. The mycolic acid, arabinogalactan, and peptidoglycan complex is composed of a cross-linked peptidoglycan, which is covalently linked to arabinogalactan chains via phosphoryl-arabinan chains. The mycobacterial cell envelope, composed exclusively of d-arabinofuranose (Ara) residues, is derived from the pentose phosphate shunt (8, 14). In keeping with this observation, it was demonstrated that DPA is, in fact, formed from 5-phosphoribose diphosphate (pRpp) (15).

Thus, the synthesis and activation of d-Ara in mycobacteria apparently follow a series of unique biosynthetic steps unlike any of those described for other organisms. Three reactions are expected: transfer of a 5-phosphopentose to decaprenyl phosphate, removal of the 5’ phosphate, and epimerization. However, the order of these steps and the identity of the enzymes catalyzing them have remained unknown. Structures of likely intermediates in the formation of DPA and three possibilities for when the epimerization occurs are illustrated in Fig. 1. We had previously speculated that the epimerization occurred at the pRpp or decaprenylphosphoryl 5-phosphoribose (DPPR) level (15). Other groups have speculated that decaprenylphosphoryl ribose (DPR) is the precursor of DPA (8, 20). The data presented here, combined with insight provided by Azorhizobium genetics, allowed definition of the pathway from pRpp to DPA in mycobacteria, including the order of events and iden-
FIG. 1. Potential intermediates in the biosynthetic pathway from pRpp to DPA in mycobacteria. The isoprenoid moiety is drawn to conform to the structure of decaprenyl phosphate identified in mycobacteria (21). Epimerization could occur between the compounds indicated by the horizontal arrows.

**METHODS AND MATERIALS**

Preparation of enzymatically active membrane and cell wall-enriched fractions. *M. smegmatis mc²155 cells* were grown in nutrient broth (EM Science) to midlog phase, harvested, washed, and stored at −70°C until required (11). Cells (10 g) were suspended in 40 ml of buffer A (50 mM morpholinepropanesulfonic acid [MOPS] [pH 7.9], 5 mM 2-mercaptoethanol, 10 mM MgCl₂), subjected to probe sonication (11), and centrifuged at 23,000 g for 20 min at 4°C. The pellet was resuspended in buffer A and Percoll (Amersham Pharmacia Biotech) was added to achieve a 60% suspension, which was centrifuged at 23,000 g for 60 min at 4°C. The white upper band, containing a particulate cell wall-enriched fraction, was isolated and Percoll was removed by repeated rounds of suspension in buffer A and centrifugation. The cell wall-enriched fraction was resuspended in buffer A to a protein concentration of 8 to 10 mg/ml for use. A membrane-enriched fraction (membrane protein) was obtained by centrifuging the 23,000 × g supernatant at 100,000 × g for 75 min at 4°C; the resulting supernatant was discarded, and the washed pellet was suspended in buffer A at a protein concentration of 15 to 20 mg/ml.

**Reaction mixtures, incubation conditions, and fractionation of reaction products.** Typical reaction mixtures for assessing p[14C]Rpp (synthesized as previously described [15] from uniformly labeled D-[14C]glucose [American Radiolabeled Chemicals, Inc.] [100 mCi/mmol]) incorporation into the lipids typically contained 1 mg of membrane protein, 60 μM ATP, 200,000 dpm of p[14C]Rpp, and buffer A in a final volume of 160 μl. Occasionally, the reaction mixture was enriched with 0.5 to 0.7 mg of the cell wall fraction; when recombinant enzymes were added 5 μg of protein was used. Reaction mixtures were incubated for the indicated times and stopped by the addition of 3 ml of CHCl₃-CH₃OH (2:1), and the products were extracted as described above.

**Cloning, expression, and purification of His-tagged Rv3790 and Rv3791.** Primers for PCR amplification of *Rv3790* and *Rv3791* were designed to include an Ndel restriction site (underlined) in both forward primers. A BamHI restriction site (underlined) as well as a stop codon were engineered into the reverse primers. Cloning, expression, and purification of His-tagged Rv3790 and Rv3791.

**Primers for PCR amplification**

- **for Rv3790/Forward**, CATATGTTGAGCGTGGGAGCT
- **for Rv3791/Forward**, CATATGGTTCTTGATGCCGTAGG
- **for Rv3791/Reverse**, GGATCCTCAGATGGGCAGCTTGCGGAAG
- **for Rv3790/Reverse**, GGATCCCTACAGCAGCTCCAAGCGTCGGGC

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**Vent DNA polymerase (Biolab, Inc.) was used to amplify M. tuberculosis H37Rv genomic DNA.** PCR products were checked for size by agarose gel electrophoresis and purified using QIAEX II agarose gel extraction kits (Qiagen). The purified DNA fragments were ligated to pSTBlue-1 by use of Perfectly Blunt cloning kits (Takara Mirus Bio Inc.) was used as the expression strain; transformed bacteria were treated with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at room tem-
perature for 4 h, harvested, resuspended in buffer A, and sonicated. The sonicate was centrifuged at 20,000 × g, the supernatant was applied to an N-nitroli- 
rack agarose (Sigma-Aldrich) column, and the tagged recombinant pro-
tected reagents were purified.

Analytical procedures. TLC analyses of enzymatically radiolabeled lipid-linked products were done using aluminum-backed silica gel 60 (F254) plates (Merck) 
developed in CHCl3-CH3OH-1 M CH3COONH4-concentrated (conc.) 
NH4OH-H2O (180:140:59:23; solvent II). Radiolabeled compounds were visualized 
by autoradiography (BioMax MR film [Kodak]). Preparative TLC of radiolabeled 
lipids was carried out under the same conditions. Radioactive bands of the 
individual lipids were visualized by autoradiography and were extracted with 1 ml 
of CHCl3-CH3OH (2:1) containing 0.05% NH4OH. Extraction of DPPR re-
quired a more polar solvent made up of water-ethanol-diethyl ether-pyridine- 
concentrated (conc.) ammonium hydroxide (15:15:5:10:017) (2). Extracts were dried under a 
stream of N2 at room temperature and dissolved in solvent I. In some cases 
chromatography on DEAE cellulose (acetate form) was used to purify radiola-
beled lipids. DEAE cellulose was packed in a Pasteur pipette and equilibrated 
with CHCl3-CH3OH (2:1); lipid extracts were dried under N2, dissolved in 
CHCl3-CH3OH (2:1), and loaded on the column. The material bound to the 
column was eluted stepwise with CHCl3-CH3OH (2:1), CH3OH, and CH3OH 
containing 10, 50, and 70 mM ammonium acetate. The partially purified lipids 
were desalted by phase partitioning (4).

Mild acid hydrolysis of the radiolabeled lipids was conducted in 50 μl of 
1-propanol-100 μl 0.02 N HCl at 60°C for 1 h. The solution was neutralized 
with 1 ml of 0.2 N NaOH, and 600 μl of CHCl3-CH3OH (2:1) was added to form 
two phases. Radioactive material in both phases was subjected to liquid scintil-
lation spectrometry and analyzed by TLC.

Mild base hydrolysis of the radiolabeled lipids was conducted in 200 μl of 
CHCl3-CH3OH (1:1) containing 0.1 M NaOH. The mixture was incubated at 
37°C for 20 min and neutralized with glacial acetic acid. CHCl3 (100 μl) and 60 
μl of H2O were added, the solution was mixed, and the resulting biphasic mixture 
was centrifuged. The upper (aqueous) phase was removed, and the lower 
(or-ganic) phase was evaporated under a stream of N2. The radiolabeled lipids 
were dissolved in solvent I, and aliquots were subjected to liquid scintillation spec-
trometry and TLC analysis.

For chemical reduction of DPX, TLC-purified material was dried under N2 
and treated with 100 μl of 1 M NH4OH in 50% ethanol containing 10 mg/ml of 
NaBH4 overnight at room temperature. Subsequently, 3 ml of CHCl3-CH3OH 
(2:1) and 0.5 ml of H2O were added, which resulted in formation of two phases. 
The lower (organic) phase was removed, dried under a stream of N2, and 
analyzed by TLC. Sugar analysis of the reduced material was performed after 
complete acid hydrolysis as described below.

In order to identify radiolabeled sugars, enzymatically labeled material was hy-
drolyzed in 2 M CF3COOH at 120°C for 1 h, dried under conditions of N2, 
washed twice with methanol, and partitioned between hexane and water. The hexane 
was removed and discarded, and the aqueous phase was dried on a rotary vacuum 
evaporator (Savant). Repeated washes of the hydrolyzed samples with deionized water 
removed residual CF3COOH. The hydrolysates were subjected to TLC anal-
ysis on silica gel 60 plates (F254; Merck) developed twice in pyridine-ethyl acetate-
glacial acetic acid-water (5:5:1:3). TLC plates were subjected to autoradiography as 
described above. Unlabeled standards were visualized by charring after spraying the 
plates with a solution of 10% cupric sulfate in 8% phosphoric acid. In some cases 
sugars were analyzed by anion exchange high-performance liquid chromatography 
on a PA-1 column as previously described (15).

Bacterial alkaline phosphatase (Invitrogen) was used to remove the phosphate 
from the 5 position of ribose 5-phosphate released from the DPPR by acid 
hydrolysis in buffer containing 10 mM TRIS-HCl (pH 8.0).

Other procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis 
analysis was performed using 12% gels; proteins were visualized with Coomassie 
blue R250. Polyvinylidene difluoride membranes were used for Western blot 
analysis, and monoclonal antipolyhistidine (mouse immunoglobulin G2a 
isotype; Sigma-Aldrich) and anti-mouse immunoglobulin G-AP conjugate (Sig-
ma-Aldrich) were used as primary and secondary antibodies. Protein concentra-
tions were estimated using a Pierce BCA kit. Restriction digests, ligations, and 
electrophoretic separations were done as described by Sambrook and Russell (13) unless 
otherwise noted. BLAST searches were done on the National Center for Bio-
technology Information website and the Mycobacterium tuberculosis Structural 
Genomics Consortium website by use of standard protein-protein BLAST 
(blastp). Alignments were done using multiple sequence alignments with hier-
archical clustering using the “Multalin” interface at the Institut National de la 
Recherche Agronomique (Toulouse, France) website.

RESULTS

As shown previously (15), when p[14C]Rpp is incubated with a particulate fraction prepared from M. smegmatis, radioactivity is rapidly incorporated into CHCl3-CH3OH (2:1) soluble material.

The incorporation of radioactivity into lipids increased linearly with time for approximately 5 min and then reached a plateau (Fig. 2A). Analysis by TLC indicated that the radioactivity was primarily incorporated into four compounds, the relative amounts of which differed over time (Fig. 2B). Three of these compounds were identified as DPPR, DPR, and DPA on the basis of previous studies (15), sensitivity to mild acid hydrolysis, and sugar analysis (Fig. 2C). A fourth compound, which ran as a somewhat diffuse band just below DPR, was stable to mild base and sensitive to mild acid hydrolysis. Thus, it had the properties of a prenyl phosphate-linked compound and was designated decaprenylphosphoryl-X.

Experiments conducted over very short time periods indicated that radioactivity derived from p[14C]Rpp is rapidly incorporated into DPPR and DPR. However, even when incubation for a period as short as 0.1 min was used it was not possible to determine which molecule was labeled first by this method. However, logic dictates the formation of DPPR was followed by dephosphorylation to DPR, a hypothesis sup-
ported by the recent identification of a DPPR synthase (7). Clearly, the compound designated DPX became labeled after 0.5 min, and significant labeling was observed in DPA after 1 min of incubation. Incorporation of radioactivity continued for periods of up to 60 min, but the amount of radioactivity in DPPR, DPR, and DPX began to decrease after 30 min. The data shown in Fig. 2 suggest that DPA derives from either DPPR or DPR and that DPX could be an intermediate.

In contrast to an earlier report (15), the radiolabeled material identified as DPPR did not contain any decaprenylphosphoryl-phosphorabinosine. This was shown by analysis of products after acid hydrolysis and alkaline phosphatase treatment. TLC analysis of the radiolabeled material after acid hydrolysis indicated the presence of a compound that comigrated with various glycosyl phosphates (Fig. 2C, lane 5; pentose phosphates do not separate on the TLC system used). However, when this compound was subsequently treated with alkaline phosphatase, a compound that comigrated with ribose was liberated with no indication of the presence of Ara (Fig. 2C, lane 6), suggesting that pRpp is not epimerized to pApp. Sugar analysis of the bands identified as DPR and DPA indicated that these compounds contained only ribose and Ara, respectively. Interestingly, acid hydrolysis of DPX did not release radioactivity that could be associated with a glucose residue by TLC analysis (Fig. 2C, lane 4).

In order to examine the possibility that epimerization occurs at the level of DPR (Fig. 1) and the precursor-product relationship of DPPR and DPR, both compounds were purified by anion exchange chromatography on DEAES cellulose and subsequently incubated with a membrane preparation from M. smegmatis. Figure 3A shows that DPPR can be enzymatically 
converted to DPR and DPA. In addition, there is a shadow just below the DPR band, suggesting that DPX may also have been formed. Figure 3B shows that the addition of purified DPR can also be enzymatically converted to DPA and perhaps DPX. However, there was no DPPR formed from
DPR. Thus, the data clearly indicate that DPPR is a precursor of DPR and importantly, in contrast to our earlier speculation (15), that DPR is a precursor of DPA.

In keeping with the fact that epimerization often involves oxidation and reduction, the addition of nicotinamide adenine dinucleotides to the reaction mixture resulted in an increase in the formation of DPA (Fig. 4A). Unexpectedly, there was little specificity for either the oxidation state of the nicotinamide adenine dinucleotides or the presence of a phosphate. That is, addition of NADPH and addition of NADH appeared to be equally effective, as did addition of NAD and addition of NADP (data not shown). Despite this lack of specificity, these results indicated that epimerization occurred via a sequential oxidation-reduction mechanism and suggested that DPX may be an oxidized intermediate such as a pentose with a keto function at C-2 or C-3. In support of this hypothesis, reduction of DPX with sodium borohydride resulted in the formation of a compound chromatographically identical to DPA (Fig. 4B). Subsequent acid hydrolysis of the chemically reduced material released a compound that is chromatographically identical to Ara (Fig. 4C), indicating that DPX is not only an intermediate

FIG. 2. Enzymatic conversion of p[14C]Rpp into [14C]DPA. (A) Incorporation of radioactivity from p[14C]Rpp into CHCl3-CH3OH (2:1) soluble material. (B) TLC analysis of enzymatically radiolabeled chloroform-methanol (2:1) soluble material. Enzymatically radiolabeled material was applied to aluminum-backed silica gel 60 F254 TLC plates (10 by 10 cm), which were developed in CHCl3–CH3OH–1 M CH3COONH4–conc. NH4OH–H2O (180:140:9:9:23). Radiolabeled compounds were visualized by autoradiography. (C) TLC analysis of sugar residues released by treatment with 2 M TFA. Lane 1, entire lipid extract; lane 2, purified DPA; lane 3, purified DPR; lane 4, purified DPX; lane 5, purified DPPR; lane 6, purified DPPR that was treated with alkaline phosphatase after acid hydrolysis. Radiolabeled compounds were visualized by autoradiography, and unlabeled standards were visualized by charring. In all cases reaction mixtures contained 1 mg of membrane-enriched protein, 60 μM ATP, and buffer A in a final volume of 160 μl. Reactions were stopped with 6 ml of CHCl3-CH3OH (2:1). Extracts were subsequently treated as described in Methods and Materials prior to liquid scintillation spectrometry or TLC analysis.

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compounds were visualized by autoradiography, and unlabeled standards were visualized by charring. In all cases reaction mixtures contained 1 mg of membrane-enriched protein, 0.6 mg of cell wall protein, 60 μM ATP, 200,000 dpm of p[14C]Rpp, and buffer A in a final volume of 160 μl. Reactions were stopped by addition of CHCl3-CH3OH (2:1). Extracts were subsequently treated as described in Methods and Materials prior to liquid scintillation spectrometry or TLC analysis.
in the formation of DPA but is also likely the immediate precursor of DPA.

Based on similarity to the Noe proteins of *Azorhizobium caulinodans* it was hypothesized that Rv3790 and Rv3791 of *M. tuberculosis* could be involved in the epimerization of ribose to Araf. Rv3790 is annotated as a probable oxidoreductase and has a flavin adenine dinucleotide (FAD) binding domain, while Rv3791 is annotated as a probable short-chain dehydrogenase that contains a FAD/NAD(P)-binding Rossmann fold (TB Structural Genomics Consortium website). Therefore, the genes encoding these proteins were cloned, expressed in *E. coli*, and subsequently purified to near homogeneity on immobilized metal affinity columns (data not shown). When both proteins were included in a reaction mixture containing FAD, NADP⁺ and NADPH, DPR was epimerized to DPA (Fig. 5A, lane 1), but no epimerization was observed when the reaction mixture contained only the enzymes, DPR, and FAD (Fig. 5A, lane 2). In the first case, formation of Ara was confirmed by sugar analysis (Fig. 5B). No conversion to DPA was observed when the enzymes were added to the reaction mixture individually.

**DISCUSSION**

The data presented here support the hypothesis that in mycobacteria, DPA is formed from pRpp as follows. First, pRpp is converted to DPPR (via a 5-phospho-o-ribose-1-diphosphate-decaprenyl-phosphate 5-phosphoribosyltransferase) (7), which is then dephosphorylated to form DPR. DPR is subsequently oxidized to form DPX followed by reduction to form DPA.

If this hypothesis is correct, it is highly probable that DPX is the product of oxidation at C-2 of DPR, specifically decaprenylphosphoryl-2-keto-β-D-erythro-pentofuranose. This conclusion is supported by the observation that chemical reduction of DPX yields DPA. One might expect the reduction to yield both DPA and DPR, but the results suggest that the trans configuration is highly favored over the cis.

A reasonable alternative is that DPR could be oxidized to form decaprenylphosphoryl-3-keto-β-D-xylo-pentofuranose. Such a compound could support epimerization at C-2; epimerization α to carbonyl groups is well known, as demonstrated in the dTDPRhamnose biosynthesis pathway (5, 6, 17). After epimerization the resulting decaprenylphosphoryl-3-keto-β-D-threo-pentofuranose could then be reduced to DPA. However, the expected products of chemical reduction of the xylo-pentofuranose would be DPR and/or decaprenylphosphoryl xylose, which were not observed. To date it has not been possible to isolate sufficient quantities of the rather unstable DPX to be able to do an unambiguous structural determination of this molecule; these studies are ongoing.

Given the probable identification of DPX as decaprenylphosphoryl-2-keto-β-D-erythro-pentofuranose it is interesting to compare the epimerization of DPR to DPA with that of UDP-glucose to UDP-galactose, which is catalyzed by GaIE. The most significant difference between the mechanism proposed here and that of GaIE is that the 2-keto pentofuranose is not a transient intermediate that remains bound to the enzyme; hence, it seemed likely that two separate enzymes for oxidation and reduction are required, as opposed to the single enzyme represented by GaIE. The reaction catalyzed by GaIE is complicated, as it requires nonstereospecificity of hydride return from the B side of the nicotinamide ring of NADH to the 4' ketopyranose intermediate; this occurs via rotation of the intermediate in the active site of the enzyme (1, 19). Active site rotation of a 2-keto pentofuranose may be more difficult than that of a 4-keto hexopyranose, as the keto group would likely move further from the site of oxidation. However, if two...
separate enzymes are utilized rotation in the active site is obviously not required.

The identification of these enzymes was aided by related work on \( \tau \)-arabinofuranosylation of the nodulation factor in \textit{A. caulinaodans}. In what began as discrete studies, two of us (M.H. and W.D.) studying \( \tau \)-arabinofuranosylation of nodulation factor in \textit{A. caulinaodans} reported that inactivation of \( \text{noeC} \) or downstream genes in \textit{A. caulinaodans} prevents \( \tau \)-arabinofuranosylation of the nodulation factor \( \text{(10)} \). Subsequently, disruption of the downstream genes \( \text{noeH}, \text{noeO}, \) and \( \text{noeP} \) was also shown to disrupt \( \tau \)-arabinofuranosylation of the nodulation factor \( \text{(data to be published elsewhere). Orthologs of} \text{noeC, noeH, and noeO (Rv3806c, Rv3790, and Rv3791, respectively) can be found in the} \text{M. tuberculosis} \) genome. Of these, \( \text{Rv3806c} \) was shown to encode a DPPR synthase \( \text{(7)} \). This work was supported by grants AI49151 (D.C.C.), AI18357 (P.J.B.), and AI33706 and R03TW200627 (M.R.M.) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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