MOLECULAR RECOGNITION AND INTERFACIAL CATALYSIS BY THE ESSENTIAL PHOSPHATIDYLINOSITOL MANNOSYLTRANSFERASE PimA FROM MYCOBACTERIA

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Mycobacterial phosphatidylinositol mannosides (PIMs) and metabolically-derived cell wall lipoglycans play important roles in host-pathogen interactions but their biosynthetic pathways are poorly understood. Here we focus on M. smegmatis PimA, an essential enzyme responsible for the initial mannosylation of phosphatidylinositol (PI). The structure of PimA in complex with GDP-mannose shows the two-domain organization and the catalytic machinery typical of GT-B glycosyltransferases. PimA is an amphitrophic enzyme that binds monodisperse PI, but its transferase activity is stimulated by high concentrations of non-substrate anionic surfactants, indicating that the early stages of PIM biosynthesis involve lipid-water interfacial catalysis. Based on structural, calorimetric and mutagenesis studies, we propose a model wherein PimA attaches to the membrane through its N-terminal domain and this association leads to enzyme activation. Our results reveal a novel mode of phosphatidylinositol recognition and provide a template for the development of potential anti-mycobacterial compounds.
The availability of mycobacterial genome sequences together with advances in the genetic manipulation of mycobacteria has led to the identification of some of the biosynthetic enzymes involved in the early stages of PIM, LM and LAM synthesis. The phosphatidylinositol mannosyltransferase (PimA, E.C. 2.4.1.57) attaches the first mannosyl residue to the 2-position of the PI inositol moiety, yielding phosphatidylinositol monomannoside (PIM₁) (14), and additional mannosyl- and acyltransferases catalyze the subsequent steps (14-17) (Figure 1). The PI synthase (8), the mannosyltransferase PimA (18) and the acyltransferase encoded by Rv2611c (G. Stadthagen & M. Jackson, unpublished results) appear to be essential for mycobacterial growth. On the other hand, the mannosyltransferases PimB and PimC that transfer the second and third mannose residues, respectively, were found to be non-essential genes, suggesting the existence of alternative downstream synthetic pathways (16). Both α-mannosyltransferases PimA and PimB use GDP-Man as a sugar donor and co-localize to a distinct sub-fraction of the plasma membrane, suggesting that the initial stages of apolar PIM synthesis take place on the cytoplasmic face of the membrane. In contrast, downstream enzymes catalyzing the synthesis of polar PIMs and LM/LAM use polyrenol-phosphate-Man (PPM) as a lipid-linked sugar donor (17,19). Furthermore, these enzymes co-localize with the plasma membrane and cell wall markers, indicating that the later stages of biosynthesis likely occur in the periplasmic space and/or the cell wall (20).

To gain insight into the molecular mechanisms that govern the early stages of PIM biosynthesis, we report here molecular and structural studies of PimA. On the basis of these data, we propose a model of interfacial catalysis wherein association of the protein with anionic membrane lipids stimulates the mannosyl transfer reaction, either by facilitating substrate diffusion to the catalytic site or by inducing an allosteric change in the enzyme's structure.

**EXPERIMENTAL PROCEDURES**

**Protein production and crystallization** – Recombinant PimA from *M. smegmatis* was produced in *E. coli*, purified to homogeneity and crystallized as described (21). The selenomethionine (SeMet) labeled protein was expressed in the *E. coli* strain BL21(DE3) pLysS (Novagen) in a M9 medium containing 0.2 g/l SeMet, and purified using the same procedure as described for the non-labeled protein. Crystals of PimA (10 mg/ml) in complex with GDP or GDP-Man (1 mM) were obtained by co-crystallization in 50 mM Hepes pH 7.5, 200 mM Ca(Ac)₂, 14% PEG 8000. Rod-like crystals appeared after 2 days and grew to a maximum size of 0.5 x 0.06 x 0.06 mm.

**Structure determination and refinement** – A three-wavelength MAD dataset (peak, inflection point and high remote) was collected to 3.5 Å resolution from a single crystal of SeMet-labeled protein in complex with GDP on the PXI beamline at the SLS (Villigen, Suisse). Single-wavelength datasets of the PimA-GDP and PimA-GDP-Man complexes were collected on beamlines ID14.1 and ID29 at the ESRF (Grenoble, France). All data were processed with programs MOSFLM, SCALA and TRUNCATE from the CCP4 program suite (22). Eight selenium sites could be identified using the program SHELXD (23) with standard settings and optimized with the program SHARP (24). The electron density map calculated with MAD phases allowed the tracing of most of the polypeptide chain, except for a few protein loops that were subsequently modeled during refinement. Data collection statistics are reported in Table 1.

The initial model was refined with CNS (25) and subsequent TLS refinements were carried out with REFMAC (26). Model building was performed with the program O (27). The GDP and GDP-Man ligands were located from the initial difference Fourier maps and manually positioned. Final refinement statistics are shown in Table 1.

**Site-Directed Mutagenesis** – The mutants PimAT9A, PimAT126W, PimAR201A, PimAE274A and PimAR77S/K78S/K80S/K81S were generated by the two-step PCR overlap method using Pfu Turbo
DNA polymerase (Stratagene) and pET-PimA as DNA template. The truncated form PimA<sub>59-70</sub> was obtained by the inverted-PCR method. The proteins were expressed in E. coli cells and purified as described for wild-type PimA (21).

Enzymatic assays – PimA activity was measured as described (18) with minor modifications: the reaction mixture contained 0.0625 µCi of GDP-[<sup>14</sup>C]-Man (specific activity, 305 mCi/mmol, Amersham Biosciences), the membrane fraction of <i>M. smegmatis</i> mc<sup>2</sup>155 (0.5 mg of proteins) providing a natural source of PI, 50 µg of purified PimA or mutated versions of PimA and 25 mM Tris-HCl buffer, pH 7.5 in a final volume 250 µl. Alternatively, mycobacterial membranes were replaced by 10 µg of PI (Sigma, bovine PI), and/or 10 µg of lyso-PI (Avanti Polar Lipids), or various amounts of 1,2-dioctanoyl-sn-glycero-3-phosphoinositol (dC8-PI) (Avanti Polar Lipids). The effect of 500 µM non-substrate surfactants was analyzed in the reaction containing 40 µM of dC8-PI. Reactions were incubated 2 h at 37 °C and stopped with 1.5 ml CHCl<sub>3</sub>/CH<sub>3</sub>OH (2:1, by vol.). The samples were left rocking 30 min at 37 °C, centrifuged at 1,500 x g for 10 min and the bottom organic phase was analyzed by TLC as described (18), or quantified by scintillation spectrometry.

Spectrophotometric assays of PimA activity employed PK/LDH enzymes. The standard PimA reaction mixture contained 50 nmol of GDP-Man (Sigma), 20 µg (23.5 nmol) of soybean PI (Sigma, added in 2 µl of 5% CHAPS), 50 µg of PimA and 50 mM Tris-HCl, pH 7.8 in the final volume of 100 µl. The mixture was incubated for 2 h at 37 °C and then immediately added to the spectrophotometric cuvette containing 240 nmol MgCl<sub>2</sub>, 240 nmol DTT, 168 nmol PEP (Sigma), 48 nmol NADH (Applichem), PK / LDH (5.9 U/8.4 U in 8.4 µl stock solution, Sigma) and 50 mM Tris-HCl, pH 7.8 in a final volume of 200 µl. Decrease in the absorbance at 340 nm was followed during the first 60 sec after addition of the PimA reaction mixture. Inhibition of PimA by glycerophosphoryl-myoinositol (GPI, Calbiochem) was assayed in the above-described assay in which the reaction mixture was supplemented with 23.5 nmols of GPI. GPI was tested also as the substrate of PimA by replacing PI with the same amount of GPI (23.5 nmol) in the reaction mixture.

Isothermal titration calorimetry – ITC was performed using a VP-ITC (MicroCal Inc.). The enzyme and the different ligands were diluted into the same batch of buffer comprising 25 mM Tris-HCl, pH 7.6 and 5% DMSO and were degassed under vacuum. Titrations were performed by injecting 25-30 consecutive aliquots (10 µl) of GDP, GDP-man, PI or GPI solutions (150 µM-1.5 mM) into the ITC cell (1.4 ml) containing wild-type PimA or point mutants (10 µM) at 25°C. ITC data were corrected for heats of dilution of substrates and product solutions. Binding stoichiometries, enthalpy values and equilibrium dissociation constants were determined by fitting corrected data to a bimolecular interaction model using Origin7 software (MicroCal Inc.).

Docking calculations – The program ICM was used for glycerophosphoryl-myoinositol (GPI) docking calculations. These calculations were performed using a stochastic global optimization procedure combined with a pseudo-Brownian positional/torsional steps and fast local gradient minimization. During the exploration of the conformational space of GPI and its relative position in the (rigid) binding pocket, 85 putative models were obtained and ranked according to the ICM score. The few highest scored models showed only small-scale variations and were further optimized by biased MC minimization using internal coordinate mechanics. The solution with the lowest calculated binding energy is shown in Figure 4A.

RESULTS

The overall structure of PimA – The crystal structures of PimA in complex with GDP and GDP-Man were determined using multiple-wavelength anomalous diffraction (MAD) methods at 2.4 Å and 2.6 Å resolution, respectively (Table 1). The enzyme, which
belongs to the ubiquitous GT-4 family of retaining glycosyltransferases (CAZY; carbohydrate-active enzymes database at http://www.cazy.org/), displays the typical GT-B fold of glycosyltransferases (28), consisting of two Rossmann-fold domains with a deep fissure at the interface that includes the catalytic center (Figure 2A). The N-terminal domain comprises residues Met1-Gly169 and Trp349-Ser373, while the C-terminal domain includes residues Val170–Asp348. The core of each domain is composed of seven parallel β-strands alternating with seven connecting α-helices. Two regions of the structure have poor or no electron density, indicating conformational flexibility: the connecting loop β3-α2 (residues 59-70) within the N-terminal domain and the C-terminal extension of the protein (residues 374-386) that is missing in other mycobacterial PimA homologs (Figure 3).

Structural homology searches using DALI (29) revealed a significant structural similarity of PimA with several GT-B enzymes belonging to different GT families (28). The closest structural neighbor is the GT-20 trehalose-6-phosphate synthase (DALI Z-score of 28.3, rmsd of 3.2 Å and 16% sequence identity), followed by the GT-4 α-1,3 glucosyltransferase WaaG (score 27.1, rmsd 3.2 Å, 16% sequence identity), the GT-72 DNA α-glucosyltransferase (score 25.4, rmsd 4.3 Å, 14% sequence identity), and the GT-5 glycogen synthase from Agrobacterium tumefaciens (score 24.7, rmsd 4.7 Å, 16% sequence identity). Significant structural matches are also found with enzymes from other GT families, such as GT-28 (PDB code 1FOK, score 21.7), GT-1 (PDB code 2CLX, score 19.3), GT-63 (PDB code 1C3J, score 18.8) and GT-35 (PDB code 2C4M, score 16.6), illustrating the overall conservation of the GT-B fold. The structure of the C-terminal domain of PimA, containing the nucleotide-binding pocket, is closely similar to those of two other GT-4 glycosyltransferases for which a crystal structure is known (30), with rmsd of 1.9-2.1 Å. More important structural differences are observed for the N-terminal domain, which is usually involved in interactions with the acceptor substrate. Among the structures deposited with the PDB, the two closest structural neighbors of the PimA N-terminal domain are those of the glycosyltransferase MurG (rmsd 2.6 Å) and the E. coli UDP-N-acetylgalactosamine epimerase (rmsd 2.7 Å).

The electrostatic surface potential of PimA (Figure 2B) reveals a polar protein with a positively charged N-terminal domain (theoretical pI 8.1) and a negatively charged C-terminal domain (theoretical pI 4.3). At the tip of the N-terminal domain, a cluster of basic residues (His76, Arg77, Lys78, Lys80 and Lys81) lies on the solvent exposed face of α-helix 2. This cluster is close to a hydrophobic surface patch and to a structurally flexible loop (residues 59-70) that is strictly conserved in mycobacterial homologs (Figure 3), suggesting the possible involvement of this region in membrane interactions. Close to the active site cleft, the N-terminal domain displays a deep pocket or tunnel (Figure 2B) that is primarily defined by conserved hydrophobic residues from strand β5 and helix α4. This tunnel might play some role in binding the fatty acid moieties of the acceptor substrate.

The substrate binding site – The sugar-donor substrate GDP-Man is clearly visible in the electron density map (Figure 2C). The guanidyl heterocycle binds to a deep pocket defined by the connecting loop β1-α1 from the N-terminal domain (residues Val13-Gly16), the ends of strands β8 and β9 and the connecting loop β10-α9 from the C-terminal domain. Several protein-ligand hydrogen-bonding interactions stabilize the donor substrate into the binding pocket (Figure 2D). In particular, the strong hydrogen bonding interactions of the guanidyl N2 nitrogen with the main-chain carbonyl group of Val251 and the carboxylate group of Asp253 may account for the nucleotide-sugar specificity of this family of enzymes (31). Ribose oxygens O2 and O3 interact with the carboxylate group of Glu282, a conserved residue in the GT-B fold superfamily (32). The distal phosphate oxygens interact with basic residues Arg196 and Lys202 and with the main-chain amide group of Gly16 from the β1-α1 loop. This loop is known to undergo significant conformational changes
upon sugar-nucleotide binding in other GT-B glycosyltransferases (33,34). The mannosyl moiety is also stabilized within the active site cleft through several hydrogen bonding contacts of the sugar ring oxygens with main-chain protein atoms and the carboxylate group of Glu274 (Figure 2D).

Although we were unable to co-crystallize PimA in complex with PI or their analogs, the 3D structure provides clear insights into the position of the polar head of the acceptor substrate within the active site. Docking calculations put the inositol moiety of PI making stacking interactions with the phenol ring of Tyr9, with its O2 atom favorably positioned to receive the mannosyl residue from GDP-Man (Figure 4A). The model predicts an important role of Arg201 in substrate binding, since its guanidinium group is well located to interact with the PI phosphate oxygens and/or the glycerol carbonyl groups. It also suggests that the hydrophobic tunnel observed in the N-terminal domain (Figure 2B) could be favorably positioned to accommodate, at least in part, the substrate acyl chains. These model predictions were assessed by site-directed mutagenesis. The functional role of Tyr9 and Arg201 in substrate binding was confirmed, since their substitution by alanine completely abolished the catalytic activity (data not shown).

The initial mannosylation of PI in mycobacteria is known to occur at the axial O2 oxygen of the inositol ring (31,36). The enzyme-substrate model described above accounts for this regio-specificity, because steric clashes of the substituent ring oxygens would preclude stacking interactions of Tyr9 with the opposite face of inositol. Furthermore, it suggests that the glycosyltransferases involved in the subsequent mannosylation of PIM (Figure 1) should display a different architecture of their substrate-binding cleft in order to mannosylate the equatorial ring oxygens of the α(1,2)-mannosyl-myo-inositol moiety. This seems to be the case for PimB, which catalyzes the transfer of the second mannosyl residue at position 6 of the inositol ring of PIM (15). Although PimA and PimB belong to the same GT4 family, they share less than 30% amino acid identity and some of the key residues in PI binding are different between the two proteins. In particular PimB lacks Tyr9 in the connecting loop β1-α1 as well as equivalent basic residues in the segment containing the RKG motif, just before α8, which includes Arg201 in PimA.

The above results suggest a plausible binding site for the polar head of the acceptor substrate. However, neither water-soluble glycerophosphoryl-myo-inositol (GPI, the fully deacylated form of PI) nor lyso-PI (a PI analog containing only one acyl chain) served as a substrate for PimA, although the latter was a competitive inhibitor of the natural substrate (Figure 4D). Binding studies using isothermal titration calorimetry (Figure 5) are consistent with these observations. GDP-Man and GDP were seen to bind PimA in enthalpy-driven reactions with dissociation constants $K_d$ of 0.23 µM ($\Delta H = -15.6$ kcal.mol$^{-1}$) and 0.03 µM ($\Delta H = -14.0$ kcal.mol$^{-1}$), respectively. On the other hand, the acceptor substrate PI interacted with the enzyme in entropy-driven reactions, and showed stronger binding to the PimA-GDP complex.
complex \( (K_d = 0.31 \mu M, \Delta H = +13.6 \text{ kcal.mol}^{-1}) \) than to ligand-free PimA \( (K_d = 2.27 \mu M, \Delta H = -1.3 \text{ kcal.mol}^{-1}) \). However, no binding of GPI could be detected either to PimA \( (K_d > 100 \mu M) \) or to the PimA-GDP complex \( (K_d > 50 \mu M) \) under the same experimental conditions (Figure 5). These results emphasize the strict requirement of the fatty acid moiety for substrate binding and catalysis.

**Protein-membrane association and interfacial catalysis –** We next investigated the binding of PI to PimA at different substrate concentrations. Surprisingly, the stoichiometry of the enzyme-substrate complex was observed to strongly depend on PI concentration (Figure 6A). The protein is able to interact not only with monodisperse PI through its active site cleft, but also with phospholipid aggregates (micelles or liposomes), possibly through a different region of the protein. Furthermore, these latter interactions were observed to stimulate the catalytic activity, as indicated by a significant increase in enzyme activity at higher PI concentrations (Figure 6B). The same behavior was observed when these experiments were carried out for a PI analog with shorter acyl chains (1,2-dioctanoyl-sn-glycerol-3-phosphoinositol), for which the critical micellar concentration (CMC) has been experimentally determined as \( 60 \pm 5 \mu M \) (37) (Figure 6C). In this case, the enzyme activity at a substrate concentration of 300 \( \mu M \) (i.e. 5 times the CMC) was over 100-fold higher than the activity measured at the CMC value. A similar increase in enzymatic activity was also observed when sub-CMC concentrations of the acceptor substrate were complemented with high concentrations of anionic non-substrate surfactants, such as cardiolipin or 1,2-dipalmitoyl-sn-glycerol-3-phosphate, but not with the zwitterionic phosphatidylethanolamine or neutral 1,2-dipalmitoyl-sn-glycerol (Figure 6D). These experiments confirm that stimulation of PimA catalysis depends on the direct association of the soluble protein with anionic membranes, in agreement with the previous observation that, although the protein was found in both membrane and cytosol fractions of a *M. smegmatis* strain overexpressing PimA, the mannosyltransferase activity was only associated with the membrane fraction (18).

Strong evidence supporting the identification of the above region as the interfacial binding surface was provided by the characterization of two PimA mutants in which the β3-α2 loop was deleted by mutagenesis (PimA_{R77S/K80S/K81S}) or the four basic residues (Arg77, Lys78, Lys80 and Lys81) on α-helix 2 were substituted by serine residues (PimA_{R77S/K78S/K80S/K81S}). These positions are far from the catalytic center and exposed to solvent (Figure 7A), and therefore they are not expected to interfere with the catalytic machinery. Indeed, the two mutants were still able to bind GDP with affinities in the sub-micromolar range (data not shown), suggesting that the integrity of the protein was not affected. However, the modifications completely inactivated PimA (Figure 7B) and drastically impaired the ability of the protein to bind phospholipid aggregates (Figure 7C, compared with Figure 6A for the wild-type protein). The involvement of the
cluster of positively charged residues in protein-membrane interactions is consistent with the observed activity enhancement of PimA in the presence of anionic, but not zwitterion, phospholipids (Figure 6D). It may also explain previous results showing that salt wash of mycobacterial plasma membranes significantly reduced the synthesis of PIM1 (40). Taken together, the above results strongly suggest that enzyme inactivation primarily arise from the disruption of protein-membrane interactions, although a putative allosteric mechanism triggered by phospholipid attachment cannot be ruled out.

**DISCUSSION**

In contrast with enzymes that process phosphorylated forms of phosphoinositides, relatively little information is known at the molecular level about proteins that handle PI as substrate or ligand. Those best characterized, such as eukaryotic lipid transfer proteins PITP (41-43) and START domains (44,45), recognize PI by encapsulating its fatty acid moieties within long tunnels running through the protein core. However, in PimA the only tunnel-like feature (Figure 2B) is smaller than those observed in eukaryotic PI-transporters, and the enzyme function is not impaired when the tunnel entrance is blocked by site-directed mutagenesis (Figure 4C). Thus, PimA likely binds PI with the fatty acid moieties only partially sequestered from the bulk solvent, probably because the phospholipid is not completely extracted from the lipid bilayer. In this sense, PimA resembles other membrane-associated lipid glycosyltransferases and particularly PigA, an ubiquitous GT-B enzyme that is part of a multi-subunit complex involved in the biosynthesis of the GPI anchor (46). Like PimA, PigA catalyzes the transfer of a sugar residue to the myo-inositol moiety of PI and requires the intact fatty acid moieties for binding and catalysis (46).

Peripheral proteins associate with single leaflets of membranes using a combination of mechanisms. These may include lipophilic attachments for membrane insertion (typically acyl chains and prenyls, though aromatic protein residues could also participate), protein domains that specifically bind to particular lipid heads (such as the pleckstrin homology or FYVE domains in phosphorylated phosphoinositide-binding proteins), hydrophobic surface patches that favor protein desolvation, and/or protein basic clusters that interact nonspecifically with acidic phospholipids (38,47). The PimA structure revealed no obvious motif that could target the enzyme to specific lipids in the membrane and there is no evidence of protein acylation or prenylation. However, deletion of the hydrophobic loop β3-α2 (which is disordered in the crystal structure) drastically impaired the interaction of the protein with PI aggregates (Figure 7C), suggesting that this loop could partially insert in the membrane and/or interact with the acyl chains of the acceptor substrate. Another major factor driving PimA-membrane association appears to be non-specific electrostatic interactions, as demonstrated by the low affinity of PimAR77S/K78S/K80S/K81S for phospholipid aggregates. Membrane-recruitment mechanisms similar to those discussed above for PimA have been put forward for other lipid glycosyltransferases, such as the bacterial glycosyltransferase MurG engaged in peptidoglycan synthesis (34) or the monoglucosyl diacylglycerol synthases involved in modulating bilayer and lipid surface properties (48). Interestingly, in these cases the N-terminal domain of the GT-B fold has also been proposed to mediate membrane interactions (34,49).

Why does interfacial binding stimulate the catalytic efficiency of PimA? Membrane association certainly facilitates substrate diffusion to the catalytic site. However, the interaction may also induce allosteric changes in the enzyme structure, as observed for example in the homologous monoglucosyl diacylglycerol synthase, where conformational changes upon liposome binding were correlated with activity increase (48). Indeed, PimA is able to bind mono-disperse PI, but has very poor or no transferase activity unless significantly higher concentrations of substrate - or other anionic surfactants - are added to the reaction mixture (Figure 6), suggesting that some conformational
changes in the protein are responsible for interfacial activation. The ITC experiments and the enzyme activity assays on wild-type and mutant forms of PimA (Figures 6,7) suggest that lipid-induced structural modifications at the N-terminal domain of the protein could directly affect binding of the acceptor substrate, for instance by facilitating the formation of a competent enzyme-substrate complex. It should be noted, however, that membrane-bound (or liposome-bound) PimA still requires the substrate acyl chains for catalysis, since deacylated PI (GPI) was not a substrate of PimA, either in the absence or in the presence of anionic surfactants (data not shown).

Our results strongly suggest that the early stages of PIM biosynthesis take place at the cytosolic side of the mycobacterial membrane. Consistent with this, the pimA gene (Rv2610c) is the fourth of a cluster of five PIM biosynthetic genes in M. tuberculosis (50), all of which are predicted to encode enzymes that interact with the membrane: PI synthase (Rv2612c) contains 6 putative transmembrane helices, and one or two transmembrane segments are predicted in the other three enzymes, namely the acyltransferase Rv2611c and two putative nucleotide hydrolases Rv2609c and Rv2613c (8,51,52). Moreover, the mannosyltransferase PimB, which transfers a second mannose residue onto PIM1, was also found to co-localize with membranes, even though this GT-B enzyme might not have a transmembrane region as suggested (15). Instead, its sequence and structural similarity with PimA suggests that PimB could use a similar mechanism for reversible membrane association. This interfacial catalytic machinery for the synthesis of apolar PIMs is probably required due to the tight association of substrates and products with the lipid bilayer, facilitating the efficient transport of the hydrophobic intermediate species along the pathway.

In summary, our results support a model of interfacial catalysis for PimA, according to which the enzyme recognizes the fully acylated substrate within the catalytic cleft and the fatty acid moieties only partially sequestered from the bulk solvent. Membrane attachment is mediated by an interfacial binding surface on the N-terminal domain of the protein, which likely includes a cluster of basic residues and the adjacent exposed loop β3-α2. Protein-membrane interactions stimulate catalysis by facilitating substrate diffusion from the lipid bilayer to the catalytic site and/or by inducing allosteric changes in the protein. The mycobacterial cell wall is the site of action of various first-line antimycobacterial agents. Since the biogenesis of PIMs is required for cell viability and it is relatively restricted to mycobacteria, the structures reported here provide a template for the design of novel chemotherapeutic agents against M. tuberculosis.

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**FOOTNOTES**

The abbreviations used are: PI, phosphatidylinositol; PIM, phosphatidylinositol mannosome; LAM, lipoarabinomannan; LM, lipomannan; SeMet, selenomethionine; GDP, guanosine diphosphate; GDP-Man, guanosinediphosphomannose; ITC, isothermal titration calorimetry; GPI, glycerophosphoryl-myo-inositol; diC4-PI, 1,2-ditetranoyl-sn-glycero-3-phosphoinositol; diC8-PI, 1,2-dioctanoyl-sn-glycero-3-phosphoinositol;

The atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 2GEJ (PimA-GDP-Man) and 2GEK (PimA-GDP).
**FIGURE LEGENDS**

**Figure 1.** Biosynthesis of PIM, LM and LAM in mycobacteria. Phosphatidylinositol mannosides (PIMs) may contain from one to six mannose residues and up to four acyl chains. PimA transfers a Manp residue from GDP-Man to the 2-position of the myo-inositol ring of PI to form PIM1.

**Figure 2.** Structure of PimA in complex with the donor substrate GDP-Man. **A.** Overall structure of PimA in complex with GDP-Man (represented as orange spheres). The topology of secondary structure elements is β1–α1–β2–α3–β4–α4–β5–α6–β6–β7–α14 for the N-terminal domain (top) and β8–α7–β9–α8–β10–α9–β11–α10–β12–α11–β13–α12–α13 for the C-terminal domain (bottom). **B.** Electrostatic charge distribution of PimA. Electronegative regions are shown in red, electropositive in blue and neutral regions in white. **C.** Final (2Fo-Fc) electron density map for GDP-Man (contoured at 1 σ). PimA residues in contact with the guanosine and ribose ring are shown in orange, those in contact with phosphates and the mannose ring in green, and those in contact with inositol phosphate in pink. **D.** Schematic view of GDP-Man – protein hydrogen bonding interactions.

**Figure 3.** Sequence alignments of mycobacterial PimA. Comparison of *Mycobacterium bovis* (Mbov, Q7TY88), *M. leprae* (Mlep, O07147), *M. smegmatis* (Msme, MSMEG2946) and *M. tuberculosis* (Mtub, O06204) PimA sequences. Secondary structure elements of PimA are shown above the protein sequence. Invariant residues are shown in an orange background. Wavy lines indicate disordered regions in the crystal structure. The basic cluster that is proposed to interact with membrane phospholipids is shown in blue. Residues involved in GP-Man binding are denoted with a full circle.

**Figure 4.** Phosphatidylinositol binding and catalysis. **A.** Molecular docking of deacylated PI into the enzyme active site. **B.** Enzymatic activities of selected PimA mutants. TLC autoradiograph of the incorporation of GDP-[14C]Man into mycobacterial membrane mannosylipids in cell free reactions. Lane 1: membranes alone, lane 2: membranes plus PimAwt, lane 3: membranes plus PimA_R201A, lane 4: membranes plus PimA_T126W, lane 5: membranes plus PimA_Y9A. **C.** TLC autoradiograph of the incorporation of GDP-[14C]Man into PI after 10 min of reaction with different concentrations of PimAwt and PimA_T126W. **D.** Activity of PimA in the presence of PI and/or lyso-PI and GPI, measured in the spectrophotometric or radioactive assay.

**Figure 5.** Isothermal titration calorimetry (ITC) measurements of enzyme-ligand interactions. The upper panel shows the raw data of the titration of PimA with GDP-man (open triangles), GDP (closed circles), GPI (closed triangles) and PI (open circles), and of the PimA-GDP complex with GPI (crossed open circles) and PI (closed squares). The lower panel shows the integrated heats of injections of the above titrations corrected for the heat of dilution and normalized to the ligand concentration. Solid lines correspond to the best fit of data using a bimolecular interaction model.

**Figure 6.** PimA interaction with lipid aggregates. **A.** Isothermal titration calorimetry measurements of the binding of substrate PI to PimA (black squares) and to the PimA-GDP complex (white squares) at low and high PI concentrations. Integrated heats of injections, corrected for the heat of dilution, are shown for 10 μl injections of a 100 μM (left panel) and 1.25 mM (right panel) PI solution into a 10 μM PimA solution. Solid lines correspond to the best fit of the data using a bimolecular interaction model. **B.** Enzyme activity as a function of PI concentration. **C.** Enzyme activity as a function of 1,2-dioctanoyl-sn-glycerol, 3-phosphoinositol (diC8-PI) concentration. **D.** Effect of non-substrate phospholipids (PE, phosphatidylethanolamine; CL, cardiolipin; DPG, 1,2-dipalmityl-sn-glycerol; and DPGP, 1,2-dipalmityl-sn-glycerol-3-phosphate) on the PimA activity using diC8-PI as a substrate.

**Figure 7.** The proposed interfacial binding surface of PimA. **A.** Optimal desolvation areas calculated on the surface of PimA. The hotspots indicate areas with the most favorable energy change upon binding,
likely to be buried in the membrane. Larger hotspots correspond to energy values lower than -15.0 kcal.mol\(^{-1}\), medium size represents values between -15 and -10 kcal.mol\(^{-1}\) and the smallest points represent values higher than -10 kcal.mol\(^{-1}\). The locations of the disordered loop (residues 59-70) and four basic residues (R77, K78, K80, K81) that could interact with membrane phospholipids are indicated. 


**C.** ITC measurements of PI binding to PimA\(_{R77S/K78S/K80S/K81S}\) (black circles), the PimA\(_{R77S/K78S/K80S/K81S}\)-GDP complex (white circles), PimA\(_{Δ59-70}\) (black triangles), and the PimA\(_{Δ59-70}\)-GDP complex (white triangles) at high substrate concentrations. Integrated heats of dilutions, corrected for the heat of dilution and normalized to the ligand concentration, are shown for 10 µl injections of a 1.25 mM PI solution into a 10 µM protein solution.
Table 1. X-ray data collection and refinement statistics.

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* Values for the highest resolution shell are shown in parenthesis.
Figure 1
Figure 2

a

b

Tunnel
Active site

180°

Basic cluster

c

d

Figure 2
Figure 4
Figure 6
Molecular recognition and interfacial catalysis by the essential phosphatidylinositol mannosyltransferase PimA from mycobacteria
Marcelo E. Guerin, Jana Kordulakova, Francis Schaeffer, Zuzana Svetlikova, Alejandro Buschiazzo, David Giganti, Brigitte Gicquel, Katarina Mikusova, Mary Jackson and Pedro M. Alzari

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