Purification and Biochemical Characterization of *Mycobacterium tuberculosis* SuhB, an Inositol Monophosphatase Involved in Inositol Biosynthesis†

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ABSTRACT: Phosphatidylinositol is an essential component of mycobacteria, and phosphatidylinositol-based lipids such as phosphatidylinositolmannosides, lipomannan, and lipoarabinomannan are major immunomodulatory components of the *Mycobacterium tuberculosis* cell wall. Inositol monophosphatase (EC 3.1.3.25) is a crucial enzyme in the biosynthesis of free *myo*-inositol from inositol-1-phosphate, a key substrate for the phosphatidylinositol synthase in mycobacteria. Analysis of the *M. tuberculosis* genome suggested the presence of four *M. tuberculosis* gene products that exhibit an inositol monophosphatase signature. In the present report, we have focused on SuhB, which possesses the highest degree of homology with human inositol monophosphatase. SuhB gene was cloned into an *E. coli* expression vector to overproduce a His-tagged protein, which was purified and characterized. SuhB required divalent metal ions for functional inositol monophosphatase activity, with Mg^{2+} being the strongest activator. Inositol monophosphatase activity catalyzed by SuhB was inhibited by the monovalent cation lithium ($IC_{50} = 0.9$ mM). As anticipated, inositol-1-phosphate was the preferred substrate ($K_m = 0.177 \pm 0.025$ mM; $k_{cat} = 3.6 \pm 0.2\ s^{-1}$); however, SuhB was also able to hydrolyze a variety of polyol phosphates such as glucitol-6-phosphate, glycerol-2-phosphate, and 2′-AMP. To provide further insight into the structure−function relationship of SuhB, different mutant proteins were generated (E83D, D104N, D107N, W234L, and D235N). These mutations almost completely abrogated inositol monophosphatase activity, thus underlining the importance of these residues in inositol-1-phosphate dephosphorylation. We also identified L81 as a key residue involved in sensitivity to lithium. The L81A mutation rendered SuhB inositol monophosphatase activity 10-fold more resistant to inhibition by lithium ($IC_{50} = 10$ mM). These studies provide the first steps in the delineation of the biosynthesis of the key metabolite inositol in *M. tuberculosis*.

Mycobacterium tuberculosis, the causative agent of tuberculosis, continues to be a problem on a worldwide scale, such that the number of mortalities attributed to the disease is estimated to be on the order of three million deaths *per annum* (*1*). The mycobacterial cell wall contains a number of constituents based on a phosphatidyl-*myo*-inositol (PI)[1] structure such as free PI, the phosphatidyl-*myo*-inositol-mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM) (*2*). These lipids play a key structural role in the cell wall of mycobacterial species but are also considered to be major immunomodulators and have been implicated in tuberculosis immunopathogenesis (*3*−*5*). Indeed, *M. tuberculosis* LAM is a virulence factor that acts as an immunosuppressive molecule inhibiting the secretion of the proinflammatory cytokines interleukin-12 (*6*, *7*) and TNF-α (*6*) and the activation of the macrophage in response to interferon-γ (*8*).

PI is ubiquitously synthesized by the PI synthase from CDP-diacylglycerol and *myo*-inositol. Salman et al. (*9*) have characterized PI biosynthesis in mycobacterial cell wall fractions, and recently Jackson et al. (*10*) have identified the *pgsA* gene of *M. tuberculosis*, which encodes the PI synthase. Moreover, it has been shown that PI is an essential component of the mycobacterial cell wall (*10*). However, little is known about the biosynthesis of the two substrates of the PI synthase (i.e., CDP-diacylglycerol and *myo*-inositol). The de novo pathway for *myo*-inositol biosynthesis involves basically a two-step process: first, cyclization of glucose-6-phosphate to inositol-1-phosphate (I-1-P) via the inositol-1-phosphate synthase (IPS) (EC 5.5.1.4) and second, dephosphorylation of inositol-1-phosphate by inositol monophosphatase (IMP) (EC 3.1.3.25) to generate inositol (*11*). Recently, using an approach based on tertiary structural prediction analysis, Bachhawat and Mande (*12*) identified an IPS (*INO1*) homologue in *M. tuberculosis* (*Rv0046c*), which was able to functionally complement an *INO1* depletion mutation in *Saccharomyces cerevisiae* (*12*). These results indicate that *M. tuberculosis Rv0046c* probably represents the mycobacterial equivalent of IPS. A *Mycobac-*

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[1] Abbreviations: Fru-1,6-diP, fructose-1,6-diphosphate; Fru-6-P, fructose-6-phosphate; Gal-6-P, galactose-6-phosphate; Glc-1-P, glucose-1-phosphate; Glc-6-P, glucose-6-phosphate; GlcA-6-P, glucuronic acid-6-phosphate; Glcol-6-P, glucitol-6-phosphate; Gly-2-P, glycerol-2-phosphate; Gly-3-P, glycerol-3-phosphate; I-1-P, inositol-1-phosphate; I-2-P, inositol-2-phosphate; IPS, inositol-1-phosphate synthase; IMP, inositol monophosphatase; LAM, lipoarabinomannan; LM, lipomannan; Manol-1-P, mannitol-1-phosphate; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PNPP, *p*-nitrophenyl phosphate.

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terium smegmatis mutant with a defective IMP gene homologue (*impA*) was also shown to possess altered cell envelope permeability properties, with a substantial reduction in the in vivo synthesis of PIM_2 (*13*). Moreover, we have recently characterized IMP activity within *M. smegmatis* cytoplasmic crude extracts (*14*). In higher eukaryotes, IMP is a Mg^{2+}-dependent enzyme, which is inhibited by lithium salts and as a consequence is the proposed biological target of lithium in the treatment of manic depression (*15*). In hyperthermophilic organisms, IMP provides inositol for the production of small solutes synthesized in response to osmotic or thermal stress (*16*). However, in some bacteria, including *Escherichia coli*, inositol-based compounds are undetectable, and the role of IMP remains unclear (*17*).

In the present study, we have focused on IMP activity that is crucial for providing *myo*-inositol to the PI synthase. Among the four *M. tuberculosis* gene products exhibiting an inositol monophosphatase signature, SuhB, which possesses the highest degree of homology with human IMP, was expressed, purified, and biochemically characterized.

METHODS AND MATERIALS

Chemicals. Chemicals (reagent grade or better), including the different SuhB substrates tested, were purchased from Sigma unless otherwise stated. D-*myo*-Inositol-1-phosphate and beryllium chloride were purchased from Fluka.

Bacterial Strains and Growth Conditions. All cloning steps were performed in *E. coli* TOP-10 (Invitrogen). Liquid cultures of recombinant *E. coli* were grown in LB broth (Merck) at 37 °C with 100 μg/mL ampicillin. *E. coli* C41 (DE3), which expresses the T7 RNA polymerase, was used as a host for the overproduction of *M. tuberculosis* SuhB.

Plasmid and DNA Manipulations. Restriction enzymes and T4 DNA ligase were purchased from Roche, and *Pfu* DNA polymerase was purchased from Promega. All DNA manipulations were performed using standards protocols, as described by Sambrook et al. (*18*).

Cloning, Expression, and Purification of M. tuberculosis SuhB in E. coli. The *SuhB* gene (*Rv2701c*) was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the following primers: *suhB*-sense, 5′-gat cga tcc ata tga cac gac ctg aca acg a-3′, and *suhB*-antisense, 5′-gat cgc ggc cgc gtc cgg gat cgg ctc tag gc-3′. The 870-base pair PCR product was purified, digested with *Nde*I and *Not*I, and ligated into pET23b (Novagen) that had been previously digested with the same enzymes. The DNA insert was sequenced to verify the absence of PCR artifacts. The resulting plasmid designated pET23b-*suhB* was used to transform *E. coli* C41 (DE3) for overproduction of the recombinant protein.

Expression and Purification of M. tuberculosis SuhB. An overnight culture of *E. coli* C41 (DE3) pET23b-*suhB* was used to inoculate a large volume of LB broth supplemented with 100 μg/mL ampicillin (Sigma) and incubated at 37 °C under shaking until the optical density (OD) at 600 nm reached 0.5. The culture was then transferred to fresh terrific broth supplemented with 100 μg/mL ampicillin and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega) overnight at 16 °C. The cells were harvested by centrifugation and resuspended in 50 mM MOPS buffer pH 7.9. Bacteria were disrupted by probe sonication (Soniprep 150, MSE Sanyo Gallenkamp, Crawley, Sussex, U.K.; 1-cm probe) for a total time of 2.5 min in 30-s pulses with 45-s cooling intervals between pulses. The resulting extracts were centrifuged at 27000*g* for 20 min at 4 °C. The supernatant was collected and applied to a Ni^{2+}-charged His-trap column (1 mL, Amersham Pharmacia Biotech) that had been equilibrated with 125 mM phosphate buffer (pH 7.5) and 0.5 M NaCl. The column was extensively washed with 50 mM imidazole in the above buffered solution and eluted with a stepwise gradient of imidazole (50−500 mM). One milliliter fractions were collected, and the presence and purity of SuhB was detected by 15% SDS-PAGE. Fractions containing pure SuhB were dialyzed against 50 mM Tris/HCl pH 7.5 and stored at −20 °C. Protein concentrations were determined using the BCA protein assay kit (Pierce; Rockford, IL).

SuhB Site-Directed Mutagenesis. Several SuhB mutant proteins were constructed using pET23b-*suhB* as a template for the QuikChange site-directed mutagenesis kit (Stratagene) with the following primers: L81A, 5′-ccc ggt gac ccg att gcc ggg gag gaa ggt-3′ and 5′-acc ttc ctc ccc ggc aat cgg gtc acc ggg 3′; E83D, 5′-gac ccg att ctc ggg gac gaa ggt ggt ggt-3′ and 5′-acc acc acc ttc gtc ccc gag aat cgg gtc-3′; D104N, 5′-gtc act tgg gtg ctc aac ccc atc gac ggc-3′ and 5′-gcc gtc gat ggg gtt gag cac cca agt gac-3′; D107N, 5′-gtg ctc gac ccc atc aac ggc acg gtg aat-3′ and 5′-att cac cgt gcc gtt gat ggg gtc gag cac-3′; W234L, 5′-cac ggg gtg cag gtg ttg gac tgt gcg gca-3′ and 5′-tgc cgc aca gtc caa cac ctg cac ccc gtg-3′; and D235N, 5′-ggg gtg cag gtg tgg aac tgt gcg gca ggt-3′ and 5′-acc tgc cgc aca gtt cca cac ctg cac ccc-3′.

Inositol Monophosphatase Assays. IMP activity was determined by quantifying the inorganic phosphate released from substrate using the Malachite Green procedure modified according to Baykov et al. (*19*). Standard assays were conducted in 100-μL reaction mixture aliquots including 50 mM Tris-HCl pH 8.5, 0.4 mM I-1-P, 10 mM Mg^{2+} and 1 μg of the purified protein. The mixture was incubated at 37 °C for 1 min. The reaction was stopped by the addition of 700 μL of water and 200 μL of the malachite green reagent. The reagent contains 2.4 M H_2SO_4, which is sufficient to stop the reaction. The release of inorganic phosphate (P_i) was determined by measuring the OD at 630 nm in comparison to that of standard P_i samples. Control assays included substrate and Mg^{2+} but no enzyme or enzyme alone. For the determination of the Mg^{2+}-dependent enzyme profile, pH optimum, K_m, metal ion dependence, monovalent cation inhibition, substrate specificity, and temperature dependence, the corresponding factors were varied. For the determination of thermal stability, the enzyme was incubated for different periods of time at 70 °C in the presence or absence of 10 mM $MgCl_2$. The activity was then measured by incubating the enzyme for 1 min at 37 °C in the presence of the substrate and 10 mM $MgCl_2$.

RESULTS

IMP Homologues in the M. tuberculosis Genome. The human IMP sequence was used to perform a BLAST search of IMP homologues in *M. tuberculosis*. The search revealed four open reading frames (ORFs) exhibiting an inositol monophosphatase signature (*20*). *Rv2701c* (*suhB*), *Rv3137*, *Rv1604* (*impA*), and *Rv2131c* (*cysQ*) exhibited 28, 25, 23,

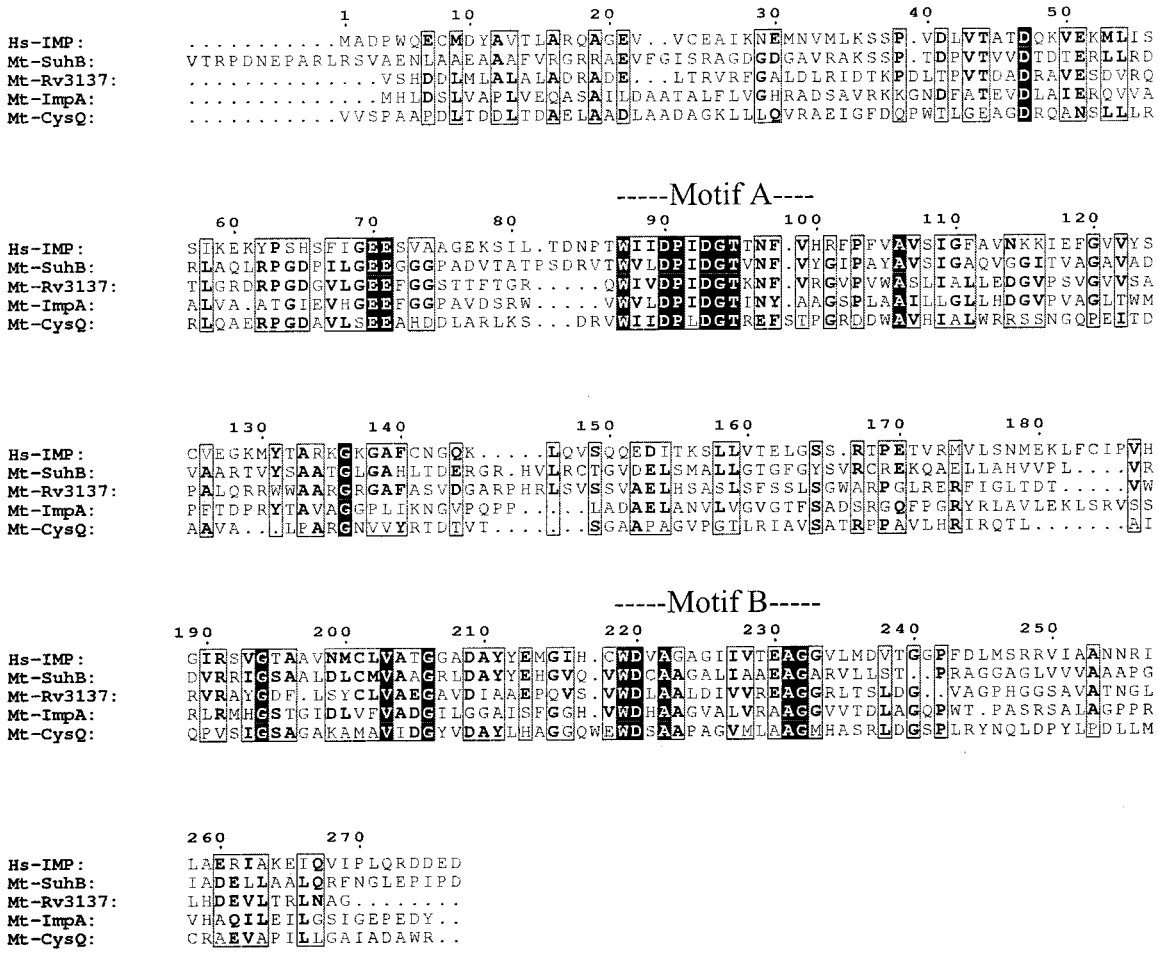

FIGURE 1: Protein sequence comparison of human (Hs) IMP and *M. tuberculosis* (Mt) homologues. Sequences alignment was performed using the MultAlin program (http://protein.toulouse.inra.fr/multalin/multalin.html) (*21*). The Figure was generated using ESPrit 2.0 software (http://prodes.toulouse.inra.fr/ESPript) (*22*).

and 19% identity, respectively and 35, 30, 29, and 26% homology with human IMP, respectively. The four ORFs shared two sequence motifs (A and B), characterized by two conservative domains DPIDGT and WDXAAG, respectively, of the super-family of lithium-sensitive phosphatases defined by Neuwald et al. (*20*) (Figure 1).

Overexpression, Purification, and Characterization of M. tuberculosis SuhB. The *M. tuberculosis* SuhB was cloned into an *E. coli* expression vector, allowing SuhB to be produced as a His-tagged protein. More than 15 mg of pure enzyme (Figure 2) was obtained from 50 mL of culture, following a one-step affinity chromatography procedure using a Ni^{2+} column. A single band on SDS-PAGE with an apparent subunit mass of around 36 kDa (Figure 2) characterized the purified SuhB protein. The value is considerably larger than the expected mass of 30 kDa, as predicted from the DNA sequence (*23*). The abnormal electrophoretic mobility of *E. coli* SuhB has already been reported (*17*), and the reason for this aberrant behavior remains unknown. In addition, this property seems to be a characteristic feature of the super-family of lithium-sensitive phosphatases because it is also shared by the rice 3′(2′),5′-diphosphonucleoside 3′(2′)-phosphohydrolase (DNPPase, RHL protein) (*24*). The oligomerization state of the protein was determined by native PAGE. A molecular mass of 93 ± 3 kDa was obtained, indicating that native *M. tuberculosis* SuhB is oligomeric, probably a trimer. However, a dimeric association is also possible because of the abnormal electrophoretic mobility of the protein, as discussed earlier. This result is in agreement with the oligomeric nature of other IMP proteins, which are usually dimers, except *E. coli* SuhB, which is active as a monomer (*17*).

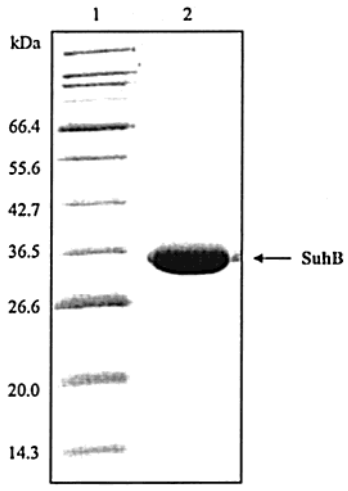

FIGURE 2: Purification of *M. tuberculosis* SuhB. SuhB was expressed as a His-tagged protein and purified by Ni^{2+}-chelate chromatography, as described in Methods and Materials. The protein was analyzed by 15% SDS-PAGE and visualized by Coomassie blue staining. Lane 1, molecular weight markers; lane 2, purified SuhB.

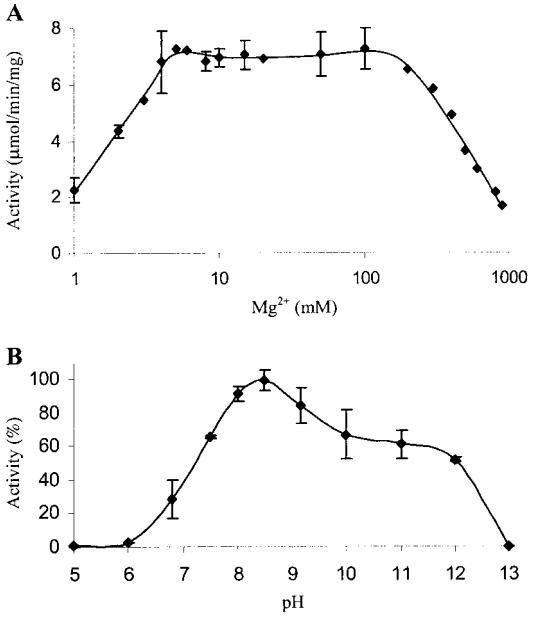

FIGURE 3: Mg^{2+} (A) and pH (B) dependence of *M. tuberculosis* SuhB IMP activity. (A) IMP activity was assayed with 0.4 mM I-1-P in 50 mM Tris-HCl, pH 8.5 at 37 °C. (B) IMP activity was assayed with 0.4 mM I-1-P in 50 mM Tris-HCl, 10 mM $MgCl_2$ at 37 °C.

Kinetic Characterization of M. tuberculosis SuhB IMP Activity. M. tuberculosis SuhB had an absolute requirement of Mg^{2+} for IMP activity. The activity increased up to a Mg^{2+} concentration of around 6 mM and remained steady until a concentration of 100 mM was reached. A further increase in Mg^{2+} concentration resulted in inhibition of IMP activity (Figure 3A). This result contrasts with the IMP activity observed for human (*25*), rat (*25*), bovine (*26*), and *E. coli* (*17*) enzymes that are inhibited at much lower concentrations of Mg^{2+} (3−5 mM) but resemble enzymes from hyperthermophilic bacteria, such as *Thermotoga maritima* (*27*), and archea bacteria, such as *Methanococcus jannaschii* (*28*) where IMP activity is stable up to 50−100 mM Mg^{2+}. The optimal pH for SuhB IMP activity was determined to be 8.5 (Figure 3B). The pH profile exhibited a dramatic drop in activity at low pH, with less than 50% activity remaining at pH 7. In contrast, the activity was extremely stable at alkaline pH, with more than 50% activity remaining at pH 12. The K_m value for I-1-P was determined to be 0.177 ± 0.025 mM. This value, taking into account the experimental errors, is within the range of K_m values determined for other known IMP enzymes (0.064−0.16 mM) (*17, 25, 27−29*). A maximum velocity of 7.2 ± 0.5 μmol/min/mg was determined at 37 °C, corresponding to a k_{cat} value of 3.6 ± 0.2 s^{-1}. The reaction velocity is similar to that of recombinant *E. coli* SuhB (6.9 μmol/min/mg) (*17*) but lower than that of recombinant human brain IMP (36.1 μmol/min/mg) (*25*).

Metal Ion Dependence for Activation and Inhibition of SuhB IMP Activity. We examined whether other divalent metal ions were able to replace Mg^{2+} in the IMP assay. Co^{2+} and Fe^{2+} at concentrations of 10 mM each gave a partial activity of around 30% of that obtained with 10 mM Mg^{2+} (Figure 4). Mn^{2+} led to a partial activity of 13%, while all the other metal ions tested gave activities weaker than 3%. Some of the cations were able to inhibit the Mg^{2+}-activated enzyme efficiently. The IC_{50} values of the different cations,

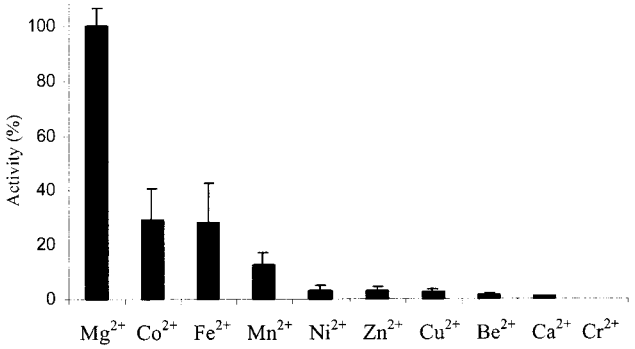

FIGURE 4: Metal ion dependence of *M. tuberculosis* SuhB IMP activity. The activities were determined using 10 mM of each cation and are normalized to the value obtained with 10 mM $MgCl_2$.

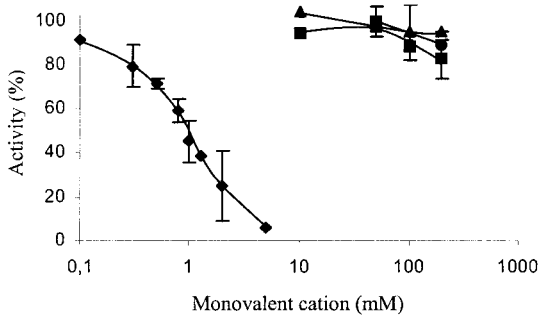

FIGURE 5: Inhibition of *M. tuberculosis* SuhB IMP activity by Li^+ (♦), Na^+ (▲), K^+ (■), and Cs^+ (●). IMP activity was assayed with 0.4 mM I-1-P in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5 at 37 °C. Activities are normalized to the value for the assay without the monovalent cation.

Table 1: IC_{50} Values of Different Metal Ions toward Mg^{2+}-Activated SuhB IMP Activity[a]

metal ion	IC_{50}
Zn^{2+}	0.5 μM
Be^{2+}	90 μM
Ca^{2+}	0.4 mM
Co^{2+}	0.7 mM
Mn^{2+}	0.8 mM
Cu^{2+}	1 mM
Cr^{2+}	3 mM
Ni^{2+}	20 mM

[a] IMP activity was assayed with 0.4 mM I-1-P in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5 at 37 °C

for the enzyme activated with 10 mM Mg^{2+}, are reported in Table 1. Zn^{2+} was the most efficient, with an IC_{50} value of 0.5 μM, followed by Be^{2+}, with an IC_{50} value of 90 μM (Table 1). It has been shown that Be^{2+} is a potent inhibitor of partially purified IMP enzymes isolated from rat brain, bovine brain, and the human neuroblastoma cell line SK-N-SH (*30*). The other metal ions exhibited IC_{50} values higher than 0.1 mM, reaching 20 mM for Ni^{2+}. Interestingly, Fe^{2+} up to a concentration of 10 mM poorly inhibited the Mg^{2+}-activated enzyme.

Inhibition by Lithium. A characteristic property of the IMP family of enzymes is inhibition by the monovalent cation Li^+. As shown in Figure 5, *M. tuberculosis* SuhB IMP activity was inhibited by Li^+, with an IC_{50} value of 0.9 mM. The value is in the same concentration range as those observed for bovine brain IMP (0.6−0.8 mM) (*26, 29*), human brain IMP (0.3−0.4 mM) (*25, 31*), and *E. coli* IMP (2 mM) (*17*) but is much higher than values obtained for

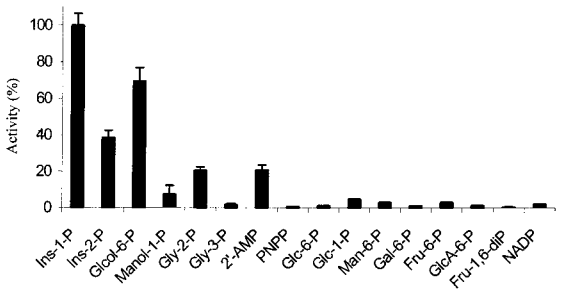

FIGURE 6: Substrate specificity of *M. tuberculosis* SuhB IMP activity. The activities were determined using 0.4 mM of each substrate and are normalized to the value obtained with 0.4 mM I-1-P. I-1-P, inositol-1-phosphate; I-2-P, inositol-2-phosphate; Glcol-6-P, glucitol-6-phosphate; Manol-1-P, mannitol-1-phosphate; Gly-2-P, glycerol-2-phosphate; Gly-3-P, glycerol-3-phosphate; PNPP, paranitrophenyl phosphate; Glc-6-P, glucose-6-phosphate; Glc-1-P, glucose-1-phosphate; Gal-6-P, galactose-6-phosphate; Fru-6-P, fructose-6-phosphate; GlcA-6-P, glucuronic acid-6-phosphate; Fru-1,6-diP, fructose-1,6-diphosphate.

plant (0.01 mM) (*32*) and yeast (0.08 mM) (*33*) IMP. The other monovalent cations tested, including Na^+, K^+, or Cs^+ had no significant effects on SuhB IMP activity between 0 and 200 mM (Figure 5).

Substrate Specificity. Among the substrates tested, I-1-P was the preferred substrate (Figure 6). Only some polyol phosphates including 2′-AMP were significantly dephosphorylated, whereas PNPP, NADP, and all the carbohydrate phosphates tested were not or were very poor substrates. I-2-P was hydrolyzed at around 40% of the rate of that of I-1-P. Surprisingly, Glcol-6-P, which was previously described as a poor substrate for *Lilium longiflorum* pollen IMP (*34*), was a very good substrate, with an activity of around 70% of that found for I-1-P. In comparison, the hydrolysis rate of Manol-1-P was 10 times lower. Gly-2-P and 2′-AMP were both hydrolyzed at 20% of the rate of hydrolysis of I-1-P, whereas Gly-3-P was virtually not a substrate. IMP enzymes have been reported to have broad substrate specificity. The thermophilic bacterial enzymes have a broad range of substrates because they are able to hydrolyze PNPP and G-1-P very efficiently (*27*, *28*). The *M. tuberculosis* SuhB IMP substrate specificity was similar to that observed for mammalian, plant, and *E. coli* IMP.

Temperature Dependence and Heat Stability of M. tuberculosis SuhB. Because *M. tuberculosis* is a human pathogen and its niche is the human body, its optimal growth temperature is 37 °C. Interestingly, *E. coli* SuhB IMP activity has been demonstrated to increase significantly with increasing temperature (*17*). Similarly, *M. tuberculosis* SuhB IMP activity was activated by temperature, with a maximum activity of 23.6 μmol/min/mg at 80 °C (Figure 7A). An Arrhenius plot of the data was linear to 60 °C (insert, Figure 7A), and the estimated activation energy of the enzyme was 9.5 kJ/mol. The enzyme itself was not very heat-stable (Figure 7B). The enzyme was preincubated for different periods of time at 70 °C, and the activity was then remeasured at 37 °C. In the absence of Mg^{2+} during the preincubation step, the enzyme was stable for 30 s but lost 50% activity after 1 min (Figure 7B). Mg^{2+} significantly improved the heat stability of the enzyme, with 96% activity remaining after a 1.5 min incubation (Figure 7B). Nevertheless, in both cases, 80% activity was lost after a 2-min incubation at 70 °C.

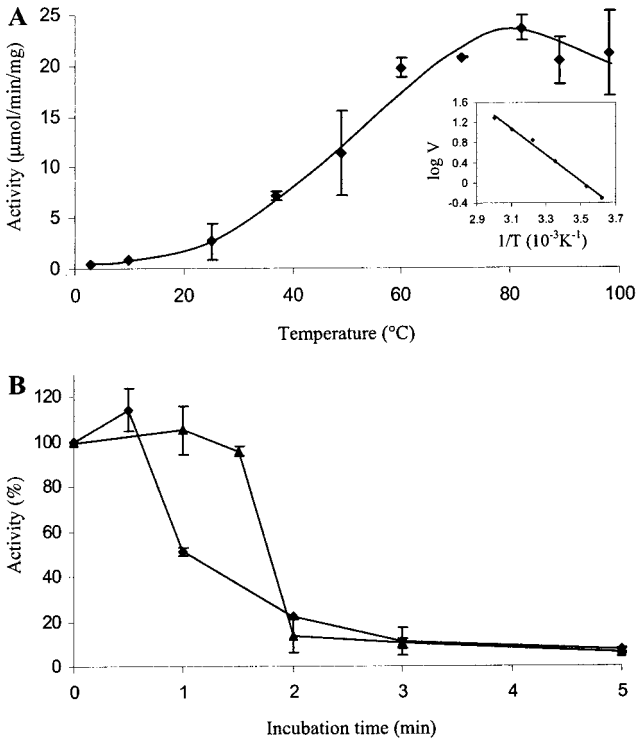

FIGURE 7: Temperature dependence (A) and thermal stability at 70 °C (B) of *M. tuberculosis* SuhB IMP activity. (A) Activity was determined at the indicated temperatures. An Arrhenius plot of the data (insert) was used to determine the activation energy, which was estimated as 9.5 kJ/mol. (B) The activity was measured at 37 °C for 1 min after incubation of the enzyme at 70 °C for various periods of time in the presence (▲) or absence (♦) of 10 mM $MgCl_2$.

Site-Directed Mutagenesis of M. tuberculosis SuhB. Structural and site-directed mutagenesis studies of human IMP have demonstrated that Glu-70, Asp-90, Asp-93, and Asp-220 bind Mg^{2+} ions, with the last two last residues also binding inositol (*31*, *35*−*38*). Furthermore, Trp-219 binds to a water molecule that stabilizes the phosphate group of inositol monophosphate. These five amino acid residues in *M. tuberculosis* SuhB correspond to Glu-83, Asp-104, Asp-107, Asp-235, and Trp-234. We have mutagenized these five residues (i.e., E83D, D104N, D107N, W234L, and D235N), and the corresponding enzymes (Figure 8A) had less than 4% activity compared with that of wild-type (WT) SuhB (Figure 8B), indicating that the function of these residues is conserved between human and *M. tuberculosis* SuhB in terms of overall IMP activity. IMP enzymes that belong to the super-family of lithium-sensitive phosphatases also include PAPases, which catalyze the hydrolysis of 3′-phosphoadenosine-5′-phosphate (PAP) to AMP (*39*). Recently, Albert et al. (*39*) determined the crystal structure of yeast PAPase (Hal2p), which is involved in salt tolerance. From a functional screen of random mutations of the HAL2 gene in growing yeast, the authors obtained enzymes with reduced cation sensitivity (*39*). Analysis of these mutants defined a salt bridge (Glu238−Arg152) and a hydrophobic bond (Val70−Trp293) as an important framework for interactions that determine cation sensitivity. The hydrophobic interaction seems to be conserved in the super-family, and the authors were able to generate a human IMP enzyme with reduced sensitivity to Li^+ through site-directed mutagenesis of the equivalent of Val-70 (i.e., Ile-68 (*39*)). We converted the equivalent of human Ile-68 in *M. tuberculosis* SuhB (i.e.,

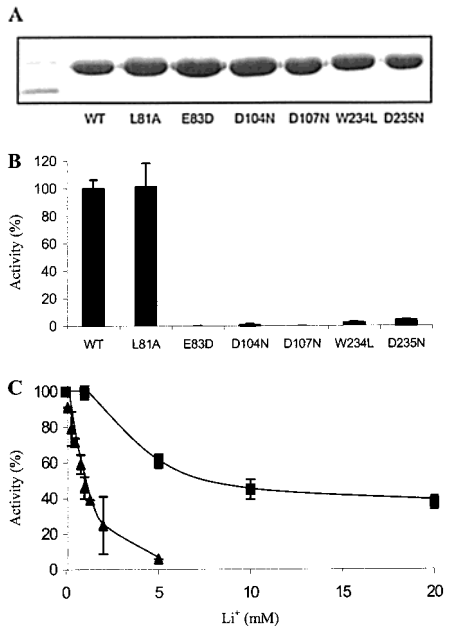

FIGURE 8: IMP activity analysis of SuhB mutants. (A) 15% SDS-PAGE analysis of SuhB wild-type (WT) and its various mutants. The molecular masses of the markers are 36.5 (top band) and 26.6 (bottom band) kDa. (B) IMP activity was assayed with 0.4 mM I-1-P in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5 at 37 °C. Activities are normalized to the activity of the wild-type protein. (C) Inhibition of SuhB WT (▲) or L81A mutant (■) IMP activity by Li^+. IMP activity was assayed with 0.4 mM I-1-P in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5 at 37 °C. Activities are normalized to the value for the assay without the monovalent cation.

Leu-81 (L81A)). The activity of the corresponding enzyme was not affected by this amino acid substitution (Figure 8B); however, the mutant enzyme was much less sensitive to inhibition by Li^+ (Figure 8C). Indeed, the IC_{50} value for Li^+ was determined to be 10 mM, as compared to 0.9 mM for wild-type SuhB IMP activity (Figure 8C), indicating that the L81A mutant enzyme is 10-fold more resistant to inhibition by Li^+ than is the wild-type enzyme. The data demonstrate that Leu-81 plays the same role in *M. tuberculosis* SuhB as do Ile-68 in human IMP and Val-70 in yeast Hal2p.

DISCUSSION

The synthesis of phosphatidylinositol in *M. smegmatis* is essential for growth (*10*). As a consequence, *myo*-inositol, which is a substrate for the PI synthase, is a critical metabolite for mycobacteria. Little is known concerning the biosynthesis of inositol in mycobacteria. The only known de novo pathway for *myo*-inositol biosynthesis involves a two-step process: first, cyclization of glucose-6-phosphate to generate *myo*-inositol-1-phosphate via inositol-1-phosphate synthase (IPS) and second, dephosphorylation of *myo*-inositol-1-phosphate by inositol monophosphatase (IMP) to yield *myo*-inositol (*11*). With the main aim to delineate inositol biosynthesis in *M. tuberculosis*, we have cloned, purified, and characterized *M. tuberculosis* SuhB, which possesses an inositol monophosphatase signature. We have characterized SuhB IMP activity with regard to pH dependence, divalent cation requirement, monovalent cation inhibition, substrate specificity, temperature dependence, and heat stability.

IMP enzymes belong to a larger super-family of phosphatases, including fructose-1,6-biphosphate 1-phosphatases (FBPases), inositol polyphosphate 1-phosphatases (IPPases), PAPases/DNPPases, and enzymes acting on both inositol-1,4-biphosphate and PAP (PIPases) that exhibit an absolute requirement for divalent metal ions and are inhibited by lithium. *M. tuberculosis* SuhB also exhibits these characteristics. In addition, *M. tuberculosis* SuhB IMP activity was very stable at alkaline pH and dramatically increased by increasing the temperature. The temperature of activation of IMP has been reported for other bacterial enzymes, such as those from the hyperthermophilic bacteria *T. maritima* and *M. jannaschii* and from *E. coli* (*17, 27, 28*). It appears that this property is characteristic of these enzymes and has been conserved during evolution, at least in bacteria.

M. tuberculosis SuhB IMP substrate specificity was similar to that observed for its mammalian, plant, and *E. coli* homologues. Nevertheless, some differences between *M. tuberculosis* and mammalian enzymes were observed. Particularly, 2′-AMP and Gly-2-P hydrolysis by *M. tuberculosis* SuhB was weaker than that described for mammalian IMP (*29*). We also report that Glcol-6-P was a good substrate with *M. tuberculosis* SuhB. Despite many similarities among IMPs of different organisms, some differences in substrate specificities are observed and remain unexplained. Chen and Roberts (*17*) found that *E. coli* SuhB substrates bear a CH−O−PO_3^{2-} group. For *M. tuberculosis* SuhB, Gly-2-P is indeed a better substrate than Gly-3-P, but Glcol-6-P possesses a methylene group; consequently, this rule cannot be applied to *M. tuberculosis* SuhB.

The sequence comparison of SuhB identified two motifs characteristic of the inositol monophosphatase family (*20*). To gain insights into the structure and function of the SuhB enzyme, we mutated several residues inferred from the sequence alignment to be involved in either Mg^{2+} (Glu-83, Asp-104, Asp-107, Asp-235) or inositol (Asp-107, Asp-235) binding. We also mutated Trp-234, which is likely to bind to a water molecule that stabilizes the phosphate group of inositol monophosphate. All the mutated enzymes had zero or <4% activity compared with that of the wild-type, indicating that these characteristic residues of the inositol monophosphatase family of enzymes are conserved in *M. tuberculosis* SuhB. We have also identified Leu-81 as a residue involved in sensitivity to Li^+. Indeed, the substitution of this residue by alanine rendered SuhB 10-fold more resistant to inhibition by Li^+. By analogy to yeast Hal2p, where sensitivity to Li^+ is mediated in part by a hydrophobic bond (Val-70−Trp-293) (*39*), we can assume that this mutation results in an altered hydrophobic interaction with Trp-234. A better understanding of the molecular basis of Li^+ sensitivity should become apparent from the crystal structures of the wild-type and the mutated enzymes. This work is currently in progress. It is noticeable that Leu-81 was conserved in *Rv3137* (Leu-65) and *Rv2131c* (*cysQ*, Leu-71) but not in *Rv1604* (*impA*) (Figure 1). A better understanding of the function of these different lithium-sensitive phosphatases and their role in *M. tuberculosis* requires the cloning, purification, and analysis of the three remaining homologues. In conclusion, this study provides the first step in the delineation of inositol biosynthesis in *M. tuberculosis*.

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