Wild-Type Phosphoribosylpyrophosphate Synthase (PRS) from Mycobacterium tuberculosis: A Bacterial Class II PRS?

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Abstract

The 5-phospho-α-D-ribose 1-diphosphate (PRPP) metabolite plays essential roles in several biosynthetic pathways, including histidine, tryptophan, nucleotides, and, in mycobacteria, cell wall precursors. PRPP is synthesized from α-D-ribose 5-phosphate (RSP) and ATP by the Mycobacterium tuberculosis prsA gene product, phosphoribosylpyrophosphate synthase (MtPRS). Here, we report amplification, cloning, expression and purification of wild-type MtPRS. Glutaraldehyde cross-linking results suggest that MtPRS predominates as a hexamer, presenting varied oligomeric states due to distinct ligand binding. MtPRS activity measurements were carried out by a novel coupled continuous spectrophotometric assay. MtPRS enzyme activity could be detected in the absence of Pi. ADP, GDP and UMP inhibit MtPRS activity. Steady-state kinetics results indicate that MtPRS has broad substrate specificity, being able to accept ATP, GTP, CTP, and UTP as diphosphoryl group donors. Fluorescence spectroscopy data suggest that the enzyme mechanism for purine diphosphoryl donors follows a random order of substrate addition, and for pyrimidine diphosphoryl donors follows an ordered mechanism of substrate addition in which RSP binds first to free enzyme. An ordered mechanism for product dissociation is followed by MtPRS, in which PRPP is the first product to be released followed by the nucleoside monophosphates products to yield free enzyme for the next round of catalysis. The broad specificity for diphosphoryl group donors and detection of enzyme activity in the absence of Pi would suggest that MtPRS belongs to Class II PRS proteins. On the other hand, the hexameric quaternary structure and allosteric ADP inhibition would place MtPRS in Class I PRSs. Further data are needed to classify MtPRS as belonging to a particular family of PRS proteins. The data here presented should help augment our understanding of MtPRS mode of action. Current efforts are toward experimental structure determination of MtPRS to provide a solid foundation for the rational design of specific inhibitors of this enzyme.

Introduction

Tuberculosis (TB) is a chronic infectious disease caused mainly by Mycobacterium tuberculosis, being the second leading cause of mortality by infectious diseases in human populations, killing about 1.7 million people worldwide in 2009 [1]. One third of the world population is estimated to be infected with latent TB. The latter is worsened by the spread of HIV-TB co-infection, which can lead to increased rates of TB reactivation, being up to 30% of deaths among HIV positive subjects caused by the TB bacilli [2]. TB infection is treated by a combination of four drugs that act upon different molecular targets [3]. The treatment regimen includes six month therapy with rifampicin and isoniazid, supplemented with pyrazinamide and ethambutol in the first two months [1]. In recent years, M. tuberculosis isolates resistant to one or more of these drugs have been spreading, which seriously hampers the success of measures to control TB [4]. The increasing incidence of TB has been paralleled by a rapid increase of cases caused by multi-drug resistant (MDR-TB) and extensively-drug resistant M. tuberculosis strains (XDR-TB), with estimated cases and annual deaths worldwide of, respectively, of 0.5 million and 100,000 for MDR-TB, and 35,000 and 20,000 for XDR-TB [5,6]. Recently, TB infection with totally resistant strains (TDR-TB), which are resistant to all first and second line classes of anti-TB drugs tested, have been isolated in Iran and India [7,8]. There is an urgent need to develop new therapeutic strategies to combat TB. Strategies based on the selection of new targets for
antimycobacterial agent development include elucidation of the role played by proteins from biochemical pathways that are essential for mycobacterial growth [9].

Phosphoribosylpyrophosphate synthase (PRS; EC 2.7.6.1) plays central roles in a number of cellular processes, catalyzing the synthesis of 5-phospho-D-ribose 1-diphosphate (PRPP; D-5-phosphoribosylpyrophosphate; D-ribose diphosphate 5-phosphate). PRS enzymes catalyze, in the presence of Mg$^{2+}$, the transfer of β,γ-diphosphoryl moiety of adenosine 5′-triphosphate (ATP) to C1-hydroxyl group of D-ribose 5-phosphate (R5P), yielding PRPP [10,11] (Figure 1). PRPP is an essential metabolite for a number of distinct biochemical pathways including de novo and salvage pathways of purine and pyrimidine nucleotide synthesis, and biosynthesis of NAD, histidine and tryptophan [12–14]. PRPP is also associated with cell integrity in Saccharomyces cerevisiae [15]. The yeast genome encodes five distinct prs genes, whose products are combined to form hetero-oligomeric catalytic active PRS, with possible role in plasma membrane stability [16]. In Corynebacteriaceae, such as mycobacteria, PRPP is a co-substrate for the synthesis of polyprenylphosphate-pentoses, which are the source of arabinosyl residues of arabinogalactan, component of the mycobacterial cell wall, and lipoarabinomannan, a highly immunogenic lipoglycan that is involved in modulating the host immune response [17,18].

PRS enzymes usually require Mg$^{2+}$-ATP as diphosphoryl group donor. The PRS proteins from Escherichia coli [19], Salmonella typhimurium [20] and mammals [21] have been shown to also require a second free Mg$^{2+}$ ion for increased catalytic rates. PRS enzymes from these organisms, as well as from Bacillus subtilis [22], are representative of Class I (also known as “Classical”) PRS proteins, with hexameric quaternary structure, allosteric inhibition by purines ribonucleoside diphosphate (adenosine 5′-diphosphate, ADP; and guanosine 5′-diphosphate, GDP), specificity for ATP (or dATP) as diphosphoryl group donor, and requirement of inorganic phosphate (Pi) for enzyme activity [23]. The three-dimensional structures of PRS enzymes from B. subtilis (PDB ID: 1IBS) [21] and Homo sapiens (PDB ID: 2H06) [11] demonstrate that the functional enzyme is a hexamer of identical subunits, associated two by two, where each monomer is composed by two domains, both with high topological similarity to the type I family of phosphoribosyltransferases [24]. In addition, there is conservation of amino acid residues in the PRP substrate binding site [22]. Class II PRS proteins share several structural characteristics with Class I enzymes. However, Class II PRSs are characterized by not being dependent on Pi for activity, have

![Figure 1. Chemical reaction catalyzed by MtPRS (Rv1017c). This figure also shows the metabolic source of RSP and the biosynthetic pathways in which the reaction product PRPP plays central roles. doi:10.1371/journal.pone.0039245.g001](image)
broad specificity for diphostate donors (including guanosine 5’-triphosphate, GTP; cytosine 5’-triphosphate, CTP; and uridine 5’-triphosphate, UTP), and are not allosterically inhibited by purines ribonucleotides diphostate [23,25]. Class II PRS proteins appear to be specific for plants as they have been identified in spinach [26] and Arabidopsis thaliana isozymes 3 and 4 [27]. Nevertheless the PRS enzyme from pathogenic Gram negative enterobacteria S. typhimurium was reported as using GTP, ITP, CTP and UTP in addition to ATP as substrate [28]. More recently, a PRS enzyme from the archaon Methanocaldococcus jannaschii has been shown to be tetrameric (PDB ID: 1U9Y), activated by P₈, non-allosterically inhibited by ADP, and that employs ATP as diphostate donor [25]. These findings prompted the proposal that M. jannaschii PRS belongs to a new Class III of PRPP synthases [25].

Here we describe cloning of prsA (Rx1017c) from M. tuberculosis; and expression, purification, molecular and kinetic characterization of the non-tagged recombinant PRS (MPrs). Glutaraldehyde cross-linking results indicate that the oligomeric state of the enzyme is predominantly hexamer in solution. However, the presence of ligands appears to stabilize alternative oligomeric states. MPrs activity was assessed by a novel coupled continuous spectrophotometric assay that measures the decrease in orotate presence of ligands appears to stabilize alternative oligomeric hydride cross-linking results indicate that the oligomeric state of the enzyme is predominantly hexamer in solution. However, the presence of ligands appears to stabilize alternative oligomeric states. MPrs activity was assessed by a novel coupled continuous spectrophotometric assay that measures the decrease in orotate concentration catalyzed by M. tuberculosis orotate phosphoribosyltransferase (MOPRT) due to PRPP formation by recombinant MPrs enzyme activity. Steady-state data indicate that MPrs can use both pyrimidine and purine nucleotides triphosphate as diphostoryl donor groups (broad specificity). In addition, enzyme activity measurements show that MPrs is catalytically competent in the absence of P₈. These data suggest that MPrs belongs to Class II PRS family, as plant homologues, even though the primary amino acid structure is indicative of structural resemblance to Class I PRS. Equilibrium binding data are also presented suggesting that MPrs mechanism is likely random order of substrate addition for purine diphostoryl donors and ordered addition of pyrimidine diphostoryl donors, with ordered release of products in which PRPP dissociation is followed by the purine or pyrimidine nucleotide monophosphate products. The prsA-encoded protein has been predicted to be essential for in vitro growth of M. tuberculosis based on transposon-site hybridization studies [29]. More recently, PRS from Corynebacterium glutamicum, a model organism used to study M. tuberculosis cell physiology, has been shown to be essential for the maintenance of cellular integrity [30]. The results presented here are discussed in light of previous reports on MPrs [30,31], and should thus contribute to a better understanding of MPrs mode of action.

Methods

Gene Amplification

The prsA gene (Rx1017c) was PCR amplified from total genomic DNA of M. tuberculosis H37Rv strain using specific primers designed to contain NdeI (primer sense 5’GGCATATGAGCCACAGCTGGGAGGTAATTCCG3’) and BamHI (primer antisense 5’GCGGATCCATGGCGTTCCCGCCTGCCAAGT3’) restriction sites (underlined). An internal restriction site for NdeI was removed from the gene sequence by site-directed mutagenesis at codon position 170, in which a thymine was replaced with a cytosine at codon’s third position (CAT to CAC), resulting in a sense mutation that maintained a histidine amino acid at this position. Dimethyl sulfoxide (DMSO) was added to the PCR reaction at final concentration of 10%. Amplified prsA gene was cloned into pET-23a(+) expression vector (Novagen). The integrity of constructs was confirmed in all cases by appropriate selections and digests with restriction enzymes (New England Biolabs). Inserted sequences were confirmed by automated DNA sequencing.

Expression and Purification of Recombinant MtPRS

Competent E. coli BL21(DE3) (Novagen) cells were electroproteinated with pET-23a(+):prsA recombinant vector and selected on Luria-Bertani (LB) agar plates containing 30 µg mL⁻¹ ampicillin. Aliquots of a 5 mL cell culture grown from a single colony were used to inoculate 500 mL of Terrific Broth (TB) medium supplemented with 50 µg mL⁻¹ ampicillin, grown at 37°C and 180 rpm to an optical density (OD₆₀₀nm) of 0.4-0.6. At this growth stage, culture temperature was lowered to 30°C and protein expression was carried out without isopropyl-β-D-thiogalactopyranoside (IPTG) induction, for 24 hours. Cells were harvested by centrifugation (11,800 g) for 30 min at 4°C and stored at −20°C. Protein purification was performed by Fast-Performance Liquid Chromatography (FPLC) on Akta Purifier System (GE HealthCare) at 4°C. Cell pellet (4 g) was suspended in 40 mL of buffer A (Tris HCl 50 mM pH 7.5) and stirred for 30 min. Cells were disrupted by sonication (12 pulses of 10 sec, with intervals of 1 min off) in presence of 0.2 mg mL⁻¹ lysozyme (Sigma Aldrich), and the clarified supernatant (48,000 g for 30 min in all cases) was further treated with 1% (wt/vol) streptomycin sulfate (Sigma-Aldrich). The latter supernatant was treated with 2.5 M ammonium sulfate and the resulting precipitate was suspended in 40 mL of buffer A (crude extract). The crude extract was loaded on a Q-Sepharose Fast Flow anion exchange column (GE Healthcare) equilibrated with buffer A. Adsorbed material was eluted with 0% to 50% linear gradient of Tris HCl 50 mM NaCl 1 M pH 7.5 (buffer B) at 1 mL min⁻¹ flow rate. Fractions containing MPrs, as inferred by 12% SDS-PAGE polyacrilamide gel electrophoresis stained with Coomassie Brilliant Blue [32], were pooled, concentrated and loaded on a Superdex 200 size exclusion column (GE Healthcare) previously equilibrated with buffer A. Proteins were eluted in isocratic conditions and fractions containing MPrs were loaded on a Mono Q HR 16/10 anion exchange column (GE Healthcare) equilibrated with buffer A. Proteins were eluted with 0% to 100% linear gradient of buffer B, at 1 mL min⁻¹ flow rate (Table 1). Homogeneous MPrs eluted at approximately 430 mM NaCl (Figure 2A). Fractions containing homogeneous MPrs were pooled, dialyzed against buffer A, concentrated, and stored at −80°C up to 7 months without any loss of enzyme activity.

MtPRS Identification by Mass Spectrometry

LC-MS/MS peptide mapping experiments were performed to confirm the identity of MPrs samples. Briefly, the purified samples were digested with trypsin using a protocol adapted from [33], and the digested peptides were chromatographically separated (Kinetex 2.6 µm C18 core-shell particles - Phenomenex, Inc.) using a nanoLC Ultra system (nanoLC Ultra 1D plus, Eksigent, USA) connected to a LTQ-Orbitrap hybrid mass spectrometer (Thermo Electron Corporation, San Jose, CA). The chromatographic method used a flow rate of 300 nL min⁻¹ with a step gradient from mobile phase A containing 0.1% formic acid in water to mobile phase B containing 0.1% formic acid in acetonitrile (0–2% B over 5 min; 2–10% B over 3 min; 10–60% B over 60 min; 60–80% B over 2 min; 80% B isocratic for 10 min; 80–2% B over 2 min; and 2% B isocratic for 8 min). MS/MS fragmentation was performed using collision-induced dissociation (CID) with an activation Q of 0.250, an activation time of 30.0 ms, 55% of normalized collision energy, and an isolation width of 1.0 Da. LC-MS/MS data were compared with theoretical MS/
the software MagTran [34] for charge state deconvolution. Of 1,000,000 charges. The average spectrum was processed with an average resolution of 30,000 at m/z 400 using FT automatic gain control target value of 50%. Orbitrap Discovery XL in profile mode at a nominal resolution range (770–2000 m/z) were collected during 20 min on a Thermo Fisher System. The electrospray source parameters were as follows: positive ion mode, 5 kV of applied voltage to the electrospray source, 37.6 V of capillary voltage, 310 V of tube lens voltage. Full spectra were acquired in the 0.3–5000 m/z range and 109 V of capillary temperature, and 109 V of tube lens voltage. Full spectra (770–2000 m/z range) were collected during 20 min on a Thermo Orbitrap Discovery XL, in profile mode at a nominal resolution of 30,000 at m/z 400 using FT automatic gain control target value of 1,000,000 charges. The average spectrum was processed with the software MagTran [34] for charge state deconvolution.

MtPRS Quaternary Structure Assessment by Cross-linking Studies

Cross-linking studies of the protein’s oligomeric states were performed as described by Fadoulouglo et al. [35], using crystallization supports with 120 μL of 25% (v/v) glutaraldehyde acidified with HCl in the reservoir. A cover slip was used to seal the reservoir, containing a 10 μL drop of protein suspension (0.5 mg mL⁻¹ homogenous recombinant MtPRS in buffer A) in its apo form and incubated with P₁, 50 mM, both in presence and absence of ATP 5 mM, R5P 5 mM, and ADP 5 mM. The plates were incubated at 30°C for different time intervals and protein drops were subsequently analyzed by 12% SDS-PAGE.

MtPRS Activity Assays

All chemicals were purchased from Sigma Aldrich. All enzyme activity assays were performed in triplicate. MtPRS activity was measured by a coupled continuous spectrophotometric assay in quartz cuvettes using a UV-visible Shimadzu spectrophotometer UV2550 equipped with a temperature-controlled cuvette holder. MtPRS reaction (ATP + R5P → PRPP + AMP) was coupled to M. tuberculosis orotate phosphoribosyltransferase (MtOPRT, EC 2.4.2.10) forward reaction (OA + PRPP → OMP + PP₃), in which PRPP synthesis can be monitored by the decrease in orotate (OA) concentration at 295 nm, for 60 sec at 25°C, using an extinction coefficient value of 3950 M⁻¹cm⁻¹ [36], when ATP was the diphosphoril group donor of the reaction catalyzed by MtPRS. When GTP, CTP and UTP were used as substrates for MtPRS enzyme activity measurements, the decrease in OA concentration was monitored at 303 nm, for 60 sec at 25°C, using an extinction coefficient of 2200 M⁻¹cm⁻¹ [37] due to strong absorption of these nucleosides triphosphate at 295 nm. Homogeneous recombinant MtOPRT was obtained as described elsewhere [38]. Coupled assay conditions so as the indicator enzyme (MtOPRT) did not limit the primary reaction (MtPRS) were employed according to [39] and [40]. The reaction mixture (500 μL) contained 1.8 U MtOPRT, 300 μM OA, 20 mM MgCl₂, in Tris HCl 50 mM pH 8.0. Reaction was started by addition of MtPRS (0.24–1.2 μM). One unit of MtPRS is defined as the amount of enzyme necessary to convert 1 μmol of R5P to PRPP per min. Effect of P₁ over MtPRS activity was assessed by varying P₁ concentration (10–50 mM) in the reaction conditions described above.

### Table 1. Purification of MtPRS from 4 g of wet cell paste of E. coli BL21(DE3) host cells.

| Purification step     | Total protein (mg) | Specific activity (U mg⁻¹) | Total enzyme activity (U) | Yield % | Purification fold |
|-----------------------|--------------------|----------------------------|---------------------------|---------|-------------------|
| crude extract         | 524                | 0.028                      | 145                       | 100     | 1                 |
| Q-Sepharose FF        | 19.6               | 0.518                      | 10.16                     | 7       | 18.5              |
| Superdex 200          | 19                 | 0.220                      | 4.17                      | 2.8     | 7.9               |
| MonoQ 16/10           | 3.5                | 1.16                       | 4.06                      | 2.8     | 41.4              |

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Apparent steady-state kinetic constants, \( K_M^{app} \) and \( V_{max}^{app} \), were determined by fitting the data for each substrate pairs to Henri-Michaelis-Menten equation, Eq. (1) [41], in which \( v_0 \), \( V_{max} \), \( S \), and \( K_M \) represent, respectively, steady-state reaction rate, maximum reaction rate, substrate concentration, and Henri-Michaelis-Menten constant for substrate \( S \). The \( k_{cat} \) values and substrate inhibition constant \( (K_I) \) were calculated, respectively, from Eq. (2) [42] and Eq. (3) [41]; in which \( k_{cat} \) and \( [E] \), correspond to, respectively, catalytic constant, or turnover number, and total enzyme concentration, for Eq. (2). For Eq. (3), \( K_i \) represents the dissociation constant for the inhibitory complex, and the remaining variables are as for Eq. (1). Data analysis was performed using SigmaPlot 10 Software.

\[
v = \frac{V_{max}[S]}{K_M + [S]} \quad (1)
\]

\[
V_{max} = k_{cat}[E]_t \quad (2)
\]

\[
v = \frac{V_{max}[S]}{K_M + [S](1 + [S]/K_I)} \quad (3)
\]

### Inhibition Assays

Inhibition assays were performed at fixed-saturating concentrations of R5P (60 μM) and ATP (300 μM), in either absence or presence of varied concentrations of ADP (20 μM to 1.5 mM), GDP (500 μM to 5 mM) or UMP (1 mM to 12 mM). Reaction was started by addition of 0.24 μM MPRS, under assay conditions described above for substrate pair ATP/R5P. All measurements were performed in triplicate. The concentration of inhibitor required to reduce the fractional enzyme activity to half of its initial value in the absence of inhibitor (IC50) was obtained from fitting the data to Eq. (4) for partial inhibition [41], in which \( y \) is the fractional activity of the enzyme in the presence of inhibitor at concentration \( [I] \); \( y_{max} \) is the maximum value of \( y \) observed at \( [I] = 0 \); and \( y_{min} \) is the minimum limiting value of \( y \) at high inhibitor concentrations.

\[
y = \frac{y_{max} - y_{min}}{1 + [I]/IC_{50}} + y_{min} \quad (4)
\]

### Intrinsic Tryptophan Fluorescence (ITF) Spectroscopy

Intrinsic tryptophan fluorescence titration was carried out to assess binary complex formation at equilibrium between MPRS and either substrate(s) or product(s) at 25°C [43]. All substrates (R5P, ATP, GTP, UTP and CTP), products (AMP and PRPP) and the enzyme were dissolved in buffer A containing MgCl2 20 mM. Fluorescence titrations were performed by making microliter additions of substrates and products at varying stock concentrations to 1 mL of MPRS 3 μM, with a maximum dilution of 6%. Ligand concentration ranges were as follow: R5P 0.99–126.83 μM; ATP 0.9–169.65 μM; GTP 0.9–309.24 μM; UTP 0.9–389.25 μM; CTP 0.9–389.25 μM; AMP 0.99–389.25 μM; and PRPP 0.99–389.25 μM. After each ligand titration, the mixture was stirred for 3 minutes to ensure equilibrium binding prior to ITF measurements. Measurements of ITF of MPRS employed excitation wavelength values of 292 nm (R5P) and 295 nm (PRPP, AMP, ATP, GTP, UTP and CTP), and the emission wavelength ranged from 300 nm to 400 nm (maximum MPRS λEM = 336 nm). In the binding experiments, different slits for, respectively, the excitation and emission wavelengths were employed: 1.5 nm and 5 nm for R5P, 1.5 nm and 10 nm for binding of ATP, GTP, UTP and CTP, and 1.5 nm and 10 nm for the products AMP and PRPP. Control experiments were performed in the same conditions in the absence of MPRS to verify any inner filter effect, and the values found in the control experiments were subtracted from those obtained in the presence of the enzyme. No corrections for effects of protein dilution on ITF upon addition of buffer A containing MgCl2 20 mM to MPRS were necessary. Data from equilibrium fluorescence spectroscopy were fitted to Eq. (5) for hyperbolic binding isotherms, in which \( F \) is the observed fluorescence signal; \( F_{max} \) is the maximal fluorescence intensity; and \( K_D \) represents the dissociation constant for binding of substrate and/or product to MPRS. Sigmoidal binding data were fitted Eq. (6) [44], in which \( F \) is the observed fluorescence signal, \( F_{max} \) is the maximal fluorescence intensity, \( n \) is the Hill coefficient, and \( K \) is a constant comprising interaction factors and the intrinsic dissociation constant [42].

\[
\frac{F}{F_{max}} = \frac{F_{max}S}{K_D + S} \quad (5)
\]

\[
\frac{F}{F_{max}} = \frac{A^n}{K^n + A^n} \quad (6)
\]

### Results

#### Cloning, Expression and Purification of Recombinant MPRS

Automated DNA sequencing confirmed the identity and integrity of the pET-23a(+):prsA construct. Recombinant MPRS protein was purified to homogeneity (Figure 2A) by a three-step chromatographic protocol, with 2.8% yield and approximately 41 fold purification (Table 1). Desorption of recombinant MPRS from Q-Sepharose Fast Flow anion exchange column occurred at approximately 390 mM salt concentration, with removal of substantial amount of contaminants from the total protein sample. Salt removal after size exclusion step led to an activity loss that was reverted after homogeneous MPRS elution from Mono Q HR at 430 mM salt concentration. Identity of recombinant MPRS was assigned by LC-MS/MS peptide mapping experiments, with coverage of 61% of its primary sequence.

### Mass Spectrometry Analyses

#### LC-MS/MS peptide mapping experiments.

Apparently homogeneous MPRS samples were desalted, digested with trypsin, and the peptide mixtures subjected to LC-MS/MS analysis as described in the Methods section. 188 spectra were obtained and identified with 27 different peptides derived from the multiple charge +6 to +8. Automated DNA sequencing confirmed the identity and integrity of the pET-23a(+):prsA construct. Recombinant MPRS protein was purified to homogeneity (Figure 2A) by a three-step chromatographic protocol, with 2.8% yield and approximately 41 fold purification (Table 1). Desorption of recombinant MPRS from Q-Sepharose Fast Flow anion exchange column occurred at approximately 390 mM salt concentration, with removal of substantial amount of contaminants from the total protein sample. Salt removal after size exclusion step led to an activity loss that was reverted after homogeneous MPRS elution from Mono Q HR at 430 mM salt concentration. Identity of recombinant MPRS was assigned by LC-MS/MS peptide mapping experiments, with coverage of 61% of its primary sequence.

#### Molecular mass determination by mass spectrometry.

The spectra of intact MPRS samples were recorded with the Orbitrap analyzer for molecular mass determination as described in the Methods section. Peaks corresponding to different charge states spanning from 25+ to the multiple charge state 45+ were detected. From the deconvoluted spectra, a value of 35,345 Da was determined for the average molecular mass of M. tuberculosis PRPP Synthase.

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**Table 1**

| Protein | Mass (Da) | Coverage (%) |
|---------|-----------|--------------|
| M. tuberculosis PRPP Synthase | 35,345 | 61% |

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**Figure 2A**

A three-step chromatographic protocol was used to purify recombinant MPRS from Q-Sepharose Fast Flow anion exchange column. The identity of the purified protein was confirmed by automated DNA sequencing, which revealed a coverage of 61% of its primary sequence.
MtPRS, consistent with the post-translational removal of the N-terminal methionine (theoretical subunit molecular mass of 33,477.47 Da with methionine and 33,346.20 without methionine) [Figure 2B]. As the value for subunit molecular mass of *E. coli* PRS is 34,219.2, the mass spectrometry analysis also demonstrates that the homogenous protein is indeed recombinant MtPRS.

**MtPRS Quaternary Structure Assignment**

MtPRS quaternary structure could not be assigned by analytical HPLC gel filtration chromatography due to formation of protein aggregates under the experimental conditions described elsewhere [45]. Cross-linking experiments of apo MtPRS were thus pursued and indicates that there is a shift from monomeric, intermediate multi-oligomeric states, to predominantly hexameric forms (approximately 220 kDa) after 45 min incubation time in the absence of Pi (Figure 3A, lanes 1, 3–5). Intermediate oligomers, mostly dimers (∼70 kDa), trimers (∼100 kDa) and tetramers (∼150 kDa) could also be visualized on Coomassie Brilliant Blue stained gels. Although the presence of Pi, 50 mM did not change this oligomerization profile, it appears to have delayed the shift of MtPRS to hexameric forms (Figure 3A, lanes 6–11). Pre-incubation with R5P 5 mM in either absence or presence of Pi, 50 mM appears to have no noticeable effects on shifting the oligomeric states of MtPRS (Figure 3B) as the profiles are similar to the apo form of MtPRS (Figure 3A). Pre-incubation with ATP 5 mM seems to stabilize MtPRS dimeric state (Figure 3C), whereas pre-incubation with ADP 5 mM suggests that there is an increase in the tetrameric state of MtPRS over time (Figure 3D), both in the absence and presence of Pi, 50 mM.

**Enzyme Activity, Substrate Specificity and Inhibition Assays**

MtPRS enzyme activity could be detected in the absence of Pi, and in the presence of varying concentrations of ATP diphosphoryl group donor at fixed 60 μM of R5P (Figure 4A). When substrate ATP was fixed at saturating concentration (300 μM) and enzyme activity measurements at varying R5P concentrations were carried out, substrate inhibition was observed at R5P concentration values larger than 60 μM (Figure 4B). Addition of 10–50 mM of Pi to the assay mixtures abrogated MtPRS enzyme activity detection due to inhibition of coupled enzyme MtOPRT (data not shown), likely due to chelating effect of PO₄³⁻ anions on Mg²⁺ cations [46]. As Mg³⁺-PRPP is the true substrate of MtOPRT [38], addition of Pi, into the reaction mixture would result in no formation of the true substrate and ensuing lack of activity of MtOPRT coupled enzyme. Accordingly, all MtPRS enzyme activity assays henceforth described were carried out in the absence of Pi.

MtPRS enzyme activity could be detected when, under the same experimental conditions, the ATP diphosphoryl group donor was replaced with either purge (GTP) or pyrimidine (CTP and UTP) nucleoside 5′-triphosphates (Figure 5). Although the values for the catalytic rate constants (k₅₆) of GTP, UTP and CTP are lower than the value for ATP, the apparent overall dissociation constant (K₃D) values are somewhat similar (Table 2). The lower k₅₆ values and similar K₃D values result in lower values for the specificity constant (k₅₆/K₃D) of GTP, UTP and CTP in comparison to ATP (Table 2). These results indicate that MtPRS has broad substrate specificity being able to use ATP, GTP, CTP and UTP as diphosphoryl group donors.

Addition of both ADP (Figure 6A) and GDP (Figure 6B) to MtPRS reaction mixture (ATP and R5P fixed at, respectively, 300 μM and 60 μM, under assay conditions described in the Methods section) resulted in partial inhibition of enzyme activity.

The data on partial enzyme inhibition were fitted to Eq. (4), yielding IC₅₀ values of, respectively, 0.07 (±0.01) mM and 0.9 (±0.1) mM for ADP and GDP. Addition UMP to the reaction mixture also resulted in partial inhibition of MtPRS, and data fitting to Eq. (4) yielded an IC₅₀ value of 3.0 (±0.3) mM (Figure 6C).

To ascertain whether or not these experimental data were due to effects on MtPRS activity and not on MtOPRT coupled enzyme, measurements of activity of the latter enzyme were performed in the presence of the diphosphoryl group donors (ATP, GTP, CTP and UTP), and nucleoside diphosphate or monophosphate inhibitors (ADP, GDP and UMP). The presence of these compounds in the assay mixtures employed in the coupled assays did not have any effect on MtOPRT enzyme activity to any extent (data not shown). Accordingly, the effects of the alternative diphosphoryl group donors, or nucleoside 5′-diphosphate or monophosphate inhibitors, were solely due to changes in MtPRS enzyme activity.

**IFT Spectroscopy**

Binary complex formation between substrates (R5P, ATP, GTP) or products (AMP, PRPP) and MtPRS was assessed by equilibrium fluorescence spectroscopy to ascertain the order (if any) of addition of these chemical compounds. Titration of MtPRS with R5P, ATP and GTP were hyperbolic (Figure 7A). These data were thus fitted to Eq. (5), yielding Kᵢ values of 61 (±3) μM for R5P, 18 (±2) μM for ATP, and 21 (±2) μM for GTP. Titration of MtPRS with AMP product was sigmoidal (Figure 7B), and data fitting to Eq. (6) yielded a value of 109 (±3) μM for Kᵢ. There was no intrinsic protein fluorescence change upon PRPP binding to MtPRS, suggesting that PRPP cannot bind to free enzyme. Binding experiments were also carried out in an attempt to determine whether or not there is binary complex formation between MtPRS and the alternative pyrimidine substrates UTP and CTP, which can also substitute for ATP as diphosphoryl group donors. No change in protein fluorescence could be detected upon titration of MtPRS enzyme with UTP and CTP.

**Discussion**

Recently, Alderwick and co-workers [30] and Lucarelli and co-workers [31] have also reported biochemical characterization of MtPRS. Both reported protocols for cloning and purification of recombinant MtPRS are significantly different from the one described herein, since MtPRS reported here was produced as a non-His-tagged protein. Although many protocols use histidine tags to facilitate protein purification by the nickel-affinity chromatography strategy, adding histidine tags may alter the protein structure and the biological activity [47,48]. We have thus deemed appropriate to make efforts to produce recombinant MtPRS without any fusion partner to avoid any possible effect that the latter may have on the former. Notwithstanding, it should be pointed out that steady-state kinetics results were shown by Lucarelli and co-workers [31] to be quite similar for His-tagged MtPRS as compared to MtPRS treated with protease for removal of the N-terminal His-tag fusion partner. The three-step chromatographic purification protocol of recombinant MtPRS here described yielded 3.5 mg of homogenous protein from 4 g of wet cell paste (Figure 2A and Table 1). Recombinant MtPRS protein was stable at −80°C in the absence of additives. However, homogeneous MtPRS could not be concentrated above 1 mg mL⁻¹ in Tris HCl 50 mM pH 7.5 without precipitation, and activity of precipitated protein could not be recovered. Interestingly, Alderwick and co-workers [30] showed that recombinant C-
Figure 3. MtPRS quaternary structure assignment by glutaraldehyde cross-linking experiments. Incubation times (numbers in black) are shown at the bottom of each lane. Underlined incubation times indicate the presence of 50 mM Pi in the reaction mixtures. Lane numbers are in white in a solid black background. M: Page Ruler Marker (Fermentas). A) Apo MtPRS. B) MtPRS incubated with R5P 5 mM. C) MtPRS incubated with ATP 5 mM. D) MtPRS incubated with ADP 5 mM.

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Figure 4. Apparent steady-state kinetic constants for MtPRS, measured under standard assay conditions (Methods), for substrate pair ATP/R5P. A) Varied ATP concentrations in presence of 60 μM R5P. B) Varied R5P concentrations in presence of saturating ATP (300 μM).

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terminal His-tagged MtPRS was stable in solution up to 2 mg mL\(^{-1}\) in KH\(_2\)PO\(_4\) buffer at pH 7.9 containing 150 mM NaCl, 1 mM DTT, 10% glycerol. It has been reported that addition of ammonium sulfate or Mg\(^{2+}\)-ATP was needed to preserve 20% of MtPRS activity in 50 mM Tris-HCl pH 8.0 and 50 mM Hepes-NaOH pH 8.0 buffers [31]. Lucarelli et al. [31] also reported that full activity of MtPRS could be maintained with addition of 50 mM Pi. No loss of activity could be observed for MtPRS in Tris HCl 50 mM pH 7.5 buffer for the protein preparation here described. The possible explanations for these conflicting experimental observations are rather elusive at the moment.

MtPRS quaternary structure could not be unequivocally determined by size exclusion liquid chromatography, in agreement with previous reports on PRS enzymes showing a tendency of these proteins to exist in multiple aggregated states in solution, ranging from dimeric to octameric quaternary structures [49,50]. Accordingly, the glutaraldehyde cross-linking method followed by SDS-PAGE analysis [35] method was employed to determine the MtPRS protein oligomerization state in solution. These data suggest that recombinant MtPRS may adopt multiple oligomeric states over time, in which the homo hexameric form is the predominant quaternary structure for apo MtPRS after 45 min incubation time in absence of Pi. Addition of 50 mM Pi to apo MtPRS seems to delay this quaternary structure organization shift from monomer to hexamer (Figure 3A). This apparent delay might be related to Pi mediated stabilization of recombinant MtPRS at alternative organization of quaternary states (in dimeric or trimeric structures), as inorganic phosphate concentration of at least 25 mM has been proposed as essential for PRS complete stability [28]. Alternatively, binding of Pi to MtPRS may lead to protection of lysine, tyrosine, histidine and arginine residues, slowing the reaction of glutaraldehyde cross-linking over time. However, whether the presence of Pi results in oligomeric state stabilization or in reduction in cross-linking remains to be established. Addition of R5P 5 mM appears to have no effect on time-dependent shifting of MtPRS oligomeric states when compared to its apo form (Figure 3B). Nevertheless, MtPRS incubation with ATP 5 mM (Figure 3C) leads to a shift towards dimeric quaternary structure with concomitant reduction in the hexameric form, whereas incubation with ADP 5 mM (Figure 3D) enhanced the MtPRS tetrameric organization. These results are in agreement with previous reports on M. tuberculosis PRPP Synthase.
recombinant PRS quaternary structure (Table 3). Sedimentation velocity experiments in 50 mM KH$_2$PO$_4$, pH 7.9 buffer containing either R5P, ATP and ADP at the same concentrations described here led to somewhat similar changes in oligomerization states of MtPRS [30]. Namely, R5P has no effect and ATP increased the hexameric species with concomitant reduction in homodimeric state of MtPRS [30]. On the other hand, ADP has been reported to affect the molar mass distribution increasing the hexameric state with a concomitant reduction in trimeric species [30]. This shift could be related to human PRS isoform 1 [11] and B. subtilis [22] ADP binding site identification on the interface of three subunits in the hexamer, a quaternary structure that might be stabilized by the presence of ADP in solution. Interestingly, analytical gel filtration results suggested that apo M. tuberculosis PRS eluted as a single symmetrical peak consistent with the hexameric state in phosphate buffer [31]. The data here presented on glutaraldehyde cross-linking (Figure 3A) and elution of a single peak from Superdex 200 size exclusion column (protein purification protocol) suggest that MtPRS exists as a hexamer in Tris HCl buffer [31]. The reason for this discrepancy is not apparent at the moment. Measurements of MtPRS enzyme activity here presented were carried out in the complete absence of ligands. MtPRS-catalyzed PRPP formation could be measured in the presence of R5P and ATP in absence of P$_i$ (Figure 4A). Interestingly, it has been reported that P$_i$ is required for MtPRS enzyme activity [30,31]. The reason for this discrepancy is not apparent at the moment. Measurements of MtPRS enzyme activity here presented were carried out in the complete absence of ligands [30]. Further efforts appear thus to be warranted to ascertain whether or not the dynamic equilibrium of MtPRS has any bearing on enzyme activity.

PRS enzyme activity is often assessed by radiochemical assays with either $^{[14]}$C-R5P [30] or $[^{32}P]$-ATP detection [11,25,49,51], by enzyme coupling with myokinase, pyruvate kinase and lactate dehydrogenase [52], or by a recently developed HPLC-based method that follows AMP formation [31]. Here we present, to the best of our knowledge, a novel coupled continuous spectrophotometric assay that measures the decrease in orotate concentration catalyzed by MtOPRT in the presence of PRPP formed in solution by MtPRS enzyme activity, a assay first proposed as a suitable alternative to follow PRS activity in the late 70’s, by Switzer and co-workers [28].

MtPRS-catalyzed PRPP formation could be measured in the presence of R5P and ATP in absence of P$_i$ (Figure 4A).

Figure 6. Inhibition of MtPRS enzyme activity by A) ADP; B) GDP; and C) UMP. MtPRS expressed as its fractional activity; and ADP, GDP and UMP concentrations were plotted on log scale.
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Figure 7. A) Hyperbolic equilibrium binding of R5P, ATP and GTP to MtPRS assessed by ITF. B) Sigmoidal equilibrium binding of AMP to MtPRS assessed by ITF.
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of Pi, since the enzyme was stored in Tris HCl 50 mM pH 7.5 and activity measurements assessed in Tris HCl 50 mM MgCl2 20 mM pH 8.0, OA 300 mM, MOPRT 1.8 U, and varied concentrations of ATP and R5P. It is possible that an explanation for this discrepancy may be attributed to coupled assay sensitivity, allowing MPRS activity detection even in absence of Pi. In addition, the steady-state kinetic constants for MPRS enzyme reported in this work (Table 2) are considerably distinct from previous reports [30,31]. It could be argued that the rather low values for the kinetic constants reported here are not representative of MPRS full activity, as it has being described that lower Pi concentrations led to partial enzyme activity [30]. However, it appears more plausible that the rather low value for the MPRS catalytic constant is due to the limiting value for the maximum velocity of the coupled enzyme (0.6 s⁻¹) as reported elsewhere [38]. Notwithstanding, the results presented here demonstrate that Pi is not an obligatory requirement for MPRS catalytic activity. An interesting feature was identification of substrate inhibition by R5P when it is varied in the presence of saturating ATP concentration (Figure 4B), with Ki value of 211 μM. Substrate inhibition by R5P has been reported for rat liver PRS [53], as well as for E. coli [54] and M. tuberculosis [30], both in the presence of non-saturating Pi concentrations. The dependence of MPRS activity upon varying Mg²⁺ concentrations could not be assessed as this cation is essential for MOPRT coupled enzyme activity [38]. We have thus fixed the Mg²⁺ concentration at 20 mM based on both the optimum concentration for activity of MOPRT (larger concentration values are inhibitory) [38] and previously reported saturation curve for the dependence of MPRS activity on increasing Mg²⁺ concentration [31]. It has been shown that the enzyme requires free Mg²⁺ as an activator and as Mg²⁺ATP co-substrate, and that free Mg²⁺ is likely to be an allosteric effector of the K-type enzyme model for cooperativity [31].

Substrate specificity measurements showed that MPRS can accept GTP, CTP, and UTP, in addition to ATP, as diphosphoryl group donors (Figure 5, Table 2), thereby showing broad substrate specificity. Although the Kₐ values are similar, the values for the catalytic rate constants of GTP, CTP and UTP are lower than ATP, MPRS main substrate. S. typhimurium PRS, another bacterial PRS, has been described as being specific for ATP, although capable of using GTP, ITP, CTP and UTP as alternative substrates to a lesser extent (3% of maximum reported activity using ATP as substrate) [28].

The purine nucleoside diphosphates ADP and GDP were reported as PRS allosteric inhibitors [23,25]. Under assay conditions here described, both behave partial inhibitors with IC₅₀ values of, respectively, 0.07±0.01 mM (Figure 6A) and 0.9±0.1 mM (Figure 6B), ADP has been shown to be a non-competitive inhibitor of MPRS with overall inhibition constant

Table 3. Comparison of biochemical data on M. tuberculosis PRS.

| Reference          | Activity assay       | [Pi] dependency | Cation dependency | Quaternary structure |
|--------------------|----------------------|-----------------|-------------------|----------------------|
| Alderwick, et al. 2011 [30] | R5P + ATP → PRPP + AMP (PRS) | 50 mM (full activation) | Activation: Mn²⁺ > Mg²⁺ | Analytical ultracentrifugation: E: trimer and hexamer (equilibrium); |
|                    | AMP + ATP → 2ADP (MK) | 5 mM (partial activity) | Inhibition: Ca²⁺ | E: RSP 5 mM: trimer and hexamer; |
|                    | ADP + PEP → 2 piruvate +2 ATP (PPK) |                      |                      | E: ATP 5 mM: ↓ hexamer ↑ dimer; |
|                    | 2 piruvate +2 NADH +2H⁺ → 2 lactate +2 NAD⁺ (LDH) |                      |                      | E: ADP 5 mM: ↓ trimer ↑ hexamer. |
|                    | 37°C | | | |
|                    | KH₂PO₄ 50 mM pH 8.0 | | | |
| Lucarelli, et al. 2010 [31] | HPLC, AMP formation | 10–40 mM (59.7 U mg⁻¹) | Activity without Pi | Size exclusion gel filtration: |
|                    | 37°C | | | Hexamer (220 KDa). |
|                    | KH₂PO₄ 50 mM pH 8.0 | | | |
| Breda, et al. 2012 (this work) | R5P + ATP → PRPP + AMP (MPRS) | Activity without Pi | – | Cross-linking: |
|                    | PRPP + OA → PP₁ + OMP (MOPRT) | (1.16 U mg⁻¹) | | E: monomer to hexamer; |
|                    | 25°C | | | E: RSP 5 mM: monomer to hexamer; |
|                    | Tris HCl 50 mM pH 8.0 | | | E: ATP 5 mM: ↓ hexamer ↑ dimer; |
|                    | | | | E: ADP 5 mM: ↑ tetramer. |

Table 4. Comparative of apparent kinetic parameters for MPRS substrate pair ATP/R5P.

| Reference          | Kinetic parameters | | | | |
|--------------------|--------------------| | | | |
| Alderwick, et al. 2011 [30] | Kₐ (μM) | Vₐₐₜ (μmol l⁻¹ min⁻¹ mg⁻¹) | k₉₅ (μL) | k₉₅/Kₐ (μL⁻¹ s⁻¹) | Kᵢ (μM) | |
|                    | 8.2 | 530 | 60.68 | 7430×10⁻¹ | – | |
| Lucarelli, et al. 2010 [31] | 71 | – | 37.1 | 521×10⁻¹ | – | |
| Breda, et al. 2012 (this work) | 14 (±2) | 1.41 (±0.07) | 0.83 (±0.04) | 59 (±8)×10⁻¹ | 211 (±28) | |
| Alderwick, et al. 2011 [30] | Kₐ = 62.65/n = 1.68 | 601 | – | – | – | |
| Lucarelli, et al. 2010 [31] | Kₐ = 260 (±50)/n = 1 | – | 34.6 (±3) | 133.1×10⁻¹ | – | |
| Breda, et al. 2012 (this work) | 25 (±4) | 1.12 (±0.03) | 0.66 (±0.02) | 26 (±4)×10⁻¹ | – | |

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M. tuberculosis PRPP Synthase
values ranging from 320 μM to 522 μM [30]. On the other hand, it has been reported an IC50 value ranging from 0.26 mM (at saturating ATP and non-saturating P_i concentrations) to 0.4 mM (saturating ATP and saturating P_i concentrations) for ADP and an IC50 larger than 5 mM for GDP inhibition of MtPRS activity in the presence of P_i [31]. These results prompted the proposal of a regulatory site to which both ADP inhibitor and P_i activator can bind [31]. Accordingly, ADP binding to the regulatory site hinderers P_i binding resulting in inhibition of MtPRS enzyme activity [31]. In addition, the sigmoidal curve for ADP inhibition of MtPRS has been shown to affect the maximum velocity only, without affecting the value of K_M and the degree of cooperativity [31]. To evaluate if MtPRS activity was responsive to pyrimidine regulation, the effect of UMP titration upon standard activity assay (Methods) was assessed (Figure 6C), which yielded an IC50 value of 3.0 (±0.8) mM. These findings seem to indicate that MtPRS activity is regulated in response to M. tuberculosis energetic demand, being more responsive to pyrimidine inhibition (ADP and GDP), although variation in pyrimidine intermediates (UMP) could also regulate its activity. The regulation of MtPRS enzyme activity by variations in both pyrimidine and purine intermediates is in accordance with the role of PRPP as a key intermediate in the metabolic pathways for the synthesis and recycling of both purine and pyrimidine nucleotides.

Hydrolytic binding isotherms determined from ITF measurements indicated that substrates R5P (K_D = 61 μM), ATP (K_D = 18 μM), and GTP (K_D = 21 μM) can bind to free apo MtPRS (Figure 7A). Dissociation constant values for ATP and GTP are somewhat similar, an indicative that there might be no substrate preference between these purine nucleosides 5′-triphosphates, which is in agreement with their similar K_M values (Table 2). Although the results of steady-state kinetic experiments have shown that CTP and UTP can act as diphosphoryl group donors, no change in ITF can be detected in the absence of R5P substrate. These findings might suggest that binding of pyrimidine nucleosides 5′-triphosphate results in no change in tryptophan fluorescence or that there is an alternative order of substrate addition for pyrimidine nucleotides. No change in ITF could be detected upon PRPP titration into free MtPRS. On the other hand, AMP product showed hyperbolic variation of ITF upon titration to free MtPRS, with K_M value of 109 μM and a Hill coefficient value of 3.2 (Figure 7B), an indicative of positive homotropic cooperativity. Further experiments of isothermal titration calorimetry could be pursued to address the issue of whether or not pyrimidine nucleotides are capable of binding to free MtPRS.

Data on steady-state kinetics and equilibrium binary complex formation suggest that the enzyme mechanism of MtPRS for purine (ATP and GTP) diphosphoryl donors follows a random-order substrate addition with ordered product dissociation, in which PRPP is the first product to be released followed by purine nucleoside monophosphate products (AMP or GMP) to yield free enzyme for the next round of catalysis (Figure 8A). Although the order of substrate addition can be proposed as it is based on solid experimental evidence, the order of product release has to be considered with caution. For instance, it is possible that PRPP binding to free MtPRS enzyme results in no change in ITF, which would imply in a random-order mechanism of product release. Isothermal titration calorimetry can thus be used to address the issue of whether or not PRPP is capable of binding to free MtPRS. The enzyme mechanism for pyrimidine (UTP or CTP) diphosphoryl donors might obey an ordered mechanism of substrate addition and product release; in which R5P binds to free enzyme followed by the diphosphorylated donors, and PRPP release is followed by pyrimidine nucleoside monophosphate products (UMP or CMP) to yield free MtPRS (Figure 8B).

Considering their molecular and kinetic characterization, three different classes of PRS enzymes have been described. Classification of PRS proteins as belonging to Class I (also known as “Classical”). Class II or Class III are based on specificity for diphosphoryl donors, requirement of P_i for activity, allosteric inhibition by purine ribonucleoside diphosphates, and oligomeric states [23,25,31]. It has been proposed that there is also a proportional relationship among K_M, V_max and PRS classes [25], in which Class III enzymes have larger K_M values for R5P and ATP substrates, Class I with the lowest values, and Class II with intermediate values [25]. The extent to which these criteria could be used for classifying PRS enzymes are still not clear due to limited number of representatives of Classes II and III PRSs [25]. MtPRS has approximately 41% identity to the human PRS isoforms, as well as to A. thaliana and spinach Class I enzymes (isoforms 1 and 2). The degree of primary sequence conservation drops to 18–23% when the M. tuberculosis sequence is compared to class II PRS enzymes from the latter two organisms (isoforms 3 and 4). As previously demonstrated [11,25,31], the amino acids involved in substrate binding are the most conserved regions: MtPRS Tyr88-Ser104 and Asp166-Arg169 for ATP binding, and MtPRS Asp219-Thr227 for R5P binding. All amino acids involved in ADP allosteric site, according to B. subtilis quaternary structure [21], are conserved in MtPRS (Ser43, Arg45, Ser77, Ala78, Lys96, His97, Arg98, Gly99, Arg100, Gln131, Asp139, His140, Ser306 and Phe311), in agreement with the inhibition data presented in
Figure 6A and with previous reports showing that ADP is an allosteric inhibitor of MtPRS [30,31]. Despite low amino acid conservation, secondary structure prediction showed that homotrimeric spinach PRS isozyme 4 (a Class II enzyme) and hexameric B. subtilis PRS (a Class I enzyme) have a similar folding pattern [25]. No Class II PRS structure has been solved so far, thus any inferences about amino acids substitution that might account for this class broader substrate specificity are, based on available structural data, somewhat speculative. PRS nucleotide binding pocket is located in a wide cleft, and the secondary structure elements might undergo conformational rearrangements upon ligand binding to accommodate both purine and pyrimidine bases, as well as properly positioning of amino acids side chains to specifically hydrogen bond each diphosphoryl group donor.

The broad specificity for diphosphoryl group donors and detection of enzyme activity in the absence of Pi would suggest that MtPRS belongs to Class II PRS proteins. On the other hand, the hexameric quaternary structure assembly, as suggested by cross-linking experiments (Figure 3) would indicate that it belongs to Class I PRS enzymes. In addition, allosteric inhibition by ADP [30,31] (Figure 6A) would place MtPRS in Class I PRSs. Accordingly, it has been previously suggested that MtPRS belongs to Class I [30]. Further data are thus needed to classify MtPRS as belonging to a particular family of PRS proteins.

It should be pointed out that the results here presented extend previous studies on MtPRS [30,31]. To the best of our knowledge, the results here presented are, along with S. typhimurium PRS data, the first experimental evidence for a bacterial PRS enzyme that can use both pyrimidine and purine nucleotides diphosphate as diphosphoryl group donors since broad substrate specificity has been described for plants only. In addition, this is the first report on MtPRS enzyme mechanism for purine and pyrimidine diphosphoryl donors. Current efforts are towards experimental structure determination of MtPRS to provide a solid foundation for the rational design of, hopefully, specific inhibitors of this enzyme without affecting to a great extent the host PRS.

Author Contributions
Conceived and designed the experiments: AB LAB DSS. Performed the experiments: AB CBB LKBM CVB LAR. Analyzed the data: AB CVB LKBM LAR. Contributed reagents/materials/analysis tools: LAB DSS. Wrote the paper: AB LAB.

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