Biochemical Analysis of the NAD\textsuperscript{+}-Dependent Malate Dehydrogenase, a Substrate of Several Serine/Threonine Protein Kinases of Mycobacterium tuberculosis

Xiao Ming Wang\textsuperscript{1}, Karine Soetaert\textsuperscript{1}, Priska Peirs\textsuperscript{1}, Michaël Kalai\textsuperscript{1}, Véronique Fontaine\textsuperscript{2}, Jean Paul Dehaye\textsuperscript{2}, Philippe Lefèvre\textsuperscript{2}\textsuperscript{*}

\textsuperscript{1} Scientific Institute of Public Health, Direction of Communicable and Infectious Diseases, Rue Engeland 642, 1180 Brussels, Belgium, \textsuperscript{2} Unité de Microbiologie Pharmaceutique et Hygiène, Faculty of Pharmacy, Université Libre de Bruxelles, Boulevard du Triomphe, CP205/2, 1050 Brussels, Belgium

* philefev@ulb.ac.be

Abstract

PknD is one of the eleven eukaryotic-like serine/threonine protein kinases (STPKs) of Mycobacterium tuberculosis (Mt\textit{b}). In vitro phosphorylation assays with the active recombinant PknD showed that the intracellular protein NAD\textsuperscript{+}-dependent malate dehydrogenase (MDH) is a substrate of this kinase. MDH, an energy-supplying enzyme, catalyzes the interconversion of malate and oxaloacetate and plays crucial roles in several metabolic pathways including the citric acid cycle. The phosphorylation site was identified on threonine residues and the phosphorylation inhibited the MDH activity. In vitro, the recombinant MDH could also be phosphorylated by at least five other STPKs, PknA, PknE, PknH, PknJ, and PknG. Immunoprecipitation analysis revealed that MDH was hyperphosphorylated in the bacteria at the beginning of the stationary and under oxygen-limited conditions by STPKs other than PknD. On the contrary, when PknD-deficient mutant mycobacteria were grown in a phosphate-depleted medium, MDH was not detectably phosphorylated. These results suggest that although the MDH is a substrate of several mycobacterial STPKs, the activity of these kinases can depend on the environment, as we identified PknD as a key element in the MDH phosphorylation assay under phosphate-poor conditions.

Introduction

Mycobacterium tuberculosis (Mt\textit{b}), a causative agent of tuberculosis, is responsible for considerable worldwide human morbidity and mortality. Nearly a third of the world population is infected with persistent or latent Mt\textit{b}, referred to as latent tuberculosis. Reactivation of latent infection is the major source of active tuberculosis in adults [1]. One of the main obstacles in the global control of tuberculosis is linked to the emergence of multi-drug resistant strains and the therapeutic failure of persistent infections treatment using conventional anti-tuberculosis...
drugs. During infection, *Mtb* resides in the hypoxic necrotic core of complex immunological structures, called granulomas, either within macrophages or in the extracellular compartment [2].

The mechanisms involved in the development of drug-tolerant bacteria are still not clear. They are thought to arise from persistent or latent bacteria with slower replication and metabolic rates. Environment induced mechanisms, such as those in response to stress (hypoxia) could lead to the development of a subpopulation of stress-tolerant cells, able to persist in a wide range of unfavorable conditions, including those where antibiotics are present [3]. It is therefore believed that the regulation of a flexible metabolism in response to environmental changes plays a significant contribution to the virulence of *Mtb* [4]. The mechanisms behind this metabolic plasticity are mostly unknown [5]. The persistent bacteria use primarily fatty acids as their carbon source [6], with the catabolism of the fatty acids releasing acetyl-CoA.

The total oxidation of the acetyl units through the tricarboxylic acid (TCA) cycle provides the bacteria with the major source of energy in aerobic pathways. Upon oxygen limitation, *Mtb* accumulates triacylglycerols (TAG) [7] and the intracellular ATP level decreases [8]. Bacterial growth arrest during mouse lung infection or nutrient starvation has been shown to be associated with increased expression and activity not only of enzymes involved in TAG synthesis, but also of isocitrate lyase and phosphoenolpyruvate carboxykinase. Concomitant with the latter, down-regulation of most TCA cycle proteins [8, 9] takes place, emphasizing the impact of anaerobic reactions and of the glyoxylate shunt that fixes carbon into biomass [10].

The two-component systems represent the classical prokaryotic mechanism for detection and response to environmental changes. Serine/threonine and tyrosine protein kinases associated with their phosphatases are also important regulatory systems in bacteria [11–13]. *Mtb* contains eleven serine/threonine protein kinases (STPKs) [12], named PknA to PknL. Two of them are soluble proteins, PknG and PknK, the nine other proteins being transmembrane kinases. Recent studies suggested extensive phosphorylation with overlapping specificity by *Mtb* STPKs [14] and reported the identification and characterization of the phosphorylation sites in substrates related to various metabolic pathways in mycobacteria [12, 14]. These include MmpL7, a transporter of polyketide virulence factors such as phthiocerol dimycocerosate [15], the anti-anti-sigma factor Rv0516c [16], the alternate SigH and SigF sigma factors, which are key regulators of the stress response [17, 18] and the transcriptional regulator VirS known to regulate the expression of the mycobacterial monoxygenase (*mymA*) operon [19]. Recently, PknA was shown to regulate the cell division via Wag31 [20]. PknE is involved in the nitric oxide stress response of *Mtb* in macrophage [21] and PknG contributes to mycobacterial survival within macrophages by preventing phagosome-lysosome fusion [22]. Noteworthy, STPK autophosphorylation not only activates the kinase domains but also creates binding sites for substrate proteins containing FHA domains, consisting in phosphothreonine-peptide recognition motifs [23–26]. Five genes coding for FHA containing proteins have been located within the *Mtb* genome, including Rv1827 encoding GarA, a *Mtb* TCA regulator [27] which is itself phosphorylated by STPKs [28], and Rv0020c encoding a protein of unknown function [29].

A further characterization of substrates of the various STPKs is critical to understand the mechanisms by which STPK-dependent phosphorylation might regulate the metabolic activity of mycobacteria, especially during the shift from aerobic to anaerobic conditions. In the present study, we focused on the *Mtb* PknD and its substrates. We showed that the NAD⁺-dependent malate dehydrogenase (MDH) was phosphorylated by several kinases including PknD. The MDH activity was reduced by the phosphorylation of the enzyme on threonine residue(s) and the phosphorylated MDH bound to the FHA containing proteins, GarA and Rv0020c. Finally, we studied the impact of environmental growth conditions on the phosphorylation of the
MDH. Using a PknD deficient mutant *Mtb* strain, we identified that PknD played a specific role under poor phosphate culture conditions.

**Material and Methods**

**Ethics statement**

The animal care and ethic committee of the "Institut Pasteur de Bruxelles" approved this study (permit number: LA1230177).

**Bacterial strains, media and chemicals**

The *E. coli* DH5α strain was used to propagate plasmids in cloning experiments, and the *E. coli* BL21 (DE3) was used to overproduce the recombinant (GST-tag) serine/threonine protein kinases, Rv1827, Rv0020c and MDH. All strains were grown and maintained in Luria-Bertani medium supplemented with 100 μg/ml ampicillin at 37°C. All restriction enzymes, T4 DNA ligase, Klenow fragment and Pfu polymerase were from Promega (Leiden, The Netherlands). Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen, Venlo, The Netherlands). Primers were purchased from Genset (Paris, France), [γ-33P]ATP and [γ-32P]ATP from Amersham Biotech (Diegem, Belgium).

*Mtb* H37Rv wild-type (WT) and the *Mtb* PknD-deficient (KO) strain [30] were cultured in Middlebrook 7H9 medium (Difco, BD Biosciences, Erembodegem, Belgium) supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco, BD Biosciences) and 0.05% Tween 80. The mutant mycobacteria were cultured in 20 μg/ml kanamycin. For the phosphate deprivation cultures, bacteria were first grown in Middlebrook 7H9 broth to an optical density at 600 nm (OD600 nm) of 0.9, washed three times with a buffer (50 mM Tris, 15 mM KCl, 10 mM (NH₄)₂SO₄, 1 mM MgSO₄ and 0.05% Tween 80, pH 7.2), resuspended in the modified Sauton medium lacking potassium dihydrogen phosphate and further incubated for 3h at 37°C. Oxygen-limited Wayne cultures were grown in Dubos Tween-albumin broth (Difco, BD Biosciences) in sealed test tubes (20 mm by 125 mm) containing a magnetic stirring bar and a head-space air ratio of 0.5 to that of medium. The tubes were placed on a magnetic stirring plate and the bars spun at a speed sufficient to keep the bacilli suspended uniformly throughout the medium without perturbing the liquid surface, as previously described [31,32]. A sterile solution of methylene blue was added to a final concentration of 1.5 μg/ml. Reduction and discoloration of this dye served as a visual indicator for oxygen depletion. *M. bovis* BCG 1173P2 (Pasteur Institute, Paris, France) was grown as a pellicle at 37°C on Sauton medium.

**Chromatographies**

*M. bovis* BCG 1173P2 cell extracts were prepared from 14-days-old cultures. The bacterial wet pellets (300 g) were suspended in 20 mM phosphate buffer, 450 mM NaCl, pH 7.3, homogenized with a potter homogenizer, then disrupted by pressure at 83–110 Mpa in a French press and clarified by centrifugation at 4,000 g. After a second centrifugation at 100,000 g for 90 min, resulting supernatants were used for hydrophobic and ion-exchange chromatographies. The soluble cell extract was applied to a phenyl-Sepharose column Cl-4B (10 x 30 cm, GE Healthcare, Diegem, Belgium), and unbound materials were recovered by washing the gel with 20 mM phosphate, 450 mM NaCl buffer, pH 7.3. A gel-filtration chromatography using a desalting column G25 (K26/10, GE Healthcare, Diegem, Belgium) replaced the phosphate buffer by 20 mM Tris pH 7.3. This solution was then loaded on a Resource-Q column (1 ml, GE Healthcare, Diegem, Belgium) and elution was performed using a 0–0.5 M NaCl gradient. The
absorbance was monitored at 280 nm. Proteins separated by the anion-exchange chromatography into different fractions were then subjected to an in vitro phosphorylation assay.

Construction and expression of His<sub>6</sub>-tagged MDH, Flag-Rv1827 and Flag-Rv0020c expression plasmids

The mdh, Rv1827 and Rv0020c genes were synthesized by PCR amplification using M. tuberculosis H37Rv genomic DNA as a template. These DNA fragments were digested with restriction enzymes and ligated in frame into vector pRSETc and pFLAG, generating pRSETc-mdh, pFLAG-Rv1827 and pFLAG-Rv0020c. The sequences were verified by DNA sequencing. The E. coli BL21(DE3) cells were transformed with the pRSETc-mdh, pFLAG-Rv1827 and pFLAG-Rv0020c vectors expressing respectively His<sub>6</sub>-MDH, Flag-Rv1827 and Flag-Rv0020c fusion proteins. These E. coli strains were grown in 200 ml at 37°C with shaking until OD<sub>600nm</sub> reached 0.6–0.8. One mM (final concentration) IPTG was then added and growth was further continued for two additional hours.

Purification of His<sub>6</sub>-MDH

Cells were harvested by centrifugation at 6,000 g for 15 min and washed twice with 20 ml Tris buffer (20 mM Tris-HCl, pH 7.5). The cell pellet was resuspended in 20 ml saline Tris buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl) and disrupted by sonication for 5 min. After centrifugation at 4°C for 30 min at 12,000 g, the supernatant containing the fusion proteins was loaded onto a 1 ml HiTrap chelating HP column (GE Healthcare, Diegem Belgium) previously saturated with 100 mM NiSO<sub>4</sub>. The His<sub>6</sub>-MDH elution was carried out in the saline Tris buffer with a 0–0.5 M imidazole gradient. Eluted fractions were analyzed by SDS-PAGE using 12% polyacrylamide gels. Pure fractions were pooled and dialyzed against a 20 mM Tris-HCl, pH 7.0, 200 mM NaCl, 1% glycerol buffer. Protein concentrations were determined by the Bradford method using bovine serum albumin as standard.

Antibody preparation and Western blot analyses

Two rabbits were injected with 0.2 ml purified MDH (1 mg/ml in PBS buffer). The injection was repeated after 4 and 7 weeks. After 10 weeks, 10 ml blood were drawn from the rabbits and stored overnight at 4°C. The clotted blood was centrifuged at 3,000 g for 10 min and the serum was collected.

Western blot analyses were carried out with different antibodies: a mAb anti-Xpress against His<sub>6</sub>-tag epitope (Invitrogen SA, Merelbeke, Belgium), a mAb anti-Flag M2 (Sigma-Aldrich BVBA, Diegem, Belgium), an anti-phosphothreonine antibody (Calbiochem, Overijse, Belgium) and anti-MDH polyclonal antibodies.

Detection of the MDH activity after non-denaturing PAGE

Proteins were separated on 8% polyacrylamide gels under non-denaturing conditions (absence of SDS from all buffers, samples were not boiled and β-mercaptoethanol was absent from the sample buffer). The MDH activity was detected by incubating the gel at 30°C in the dark in 15 ml of 100 mM Tris/acetate, pH 8.0 containing 25 mM L-malate, 0.22 mM NAD, 0.005% phenazine methosulfate and 0.008% nitro blue tetrazolium, as previously described by Cannata and Cazzulo [33].
**In vitro phosphorylation assay**

One μg protein was incubated at 37°C for 30 min with 250 ng of recombinant GST-PknD in the presence of 5 μCi [γ-33P]ATP in a 25 mM Heps, 60 mM KCl, 1 mM DTT, 2.5 mM Mn acetate (pH 7.5) buffer. The products of the reactions were then separated by SDS-PAGE (12% polyacrylamide gels). The gels were dried and exposed on X-ray film (Kodak XAR) for autoradiography.

**Analysis of the N-terminal amino acids sequence**

Proteins were transferred from the SDS-PAGE gels to polyvinylidene difluoride (PVDF) membranes using a LKB Multiphor II electrophoresis unit by semidyry electroblotting. N-terminal amino acids sequence analysis was performed by Edman degradation in an automated 476-A pulsed liquid sequencer equipped with an on-line PTH-amino acid analyzer (Applied Biosystems, Foster City, CA, USA).

**Identification of the phosphorylated amino acid in MDH**

The 32P-phosphorylated protein samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Phosphorylated proteins bound to the membrane were detected by autoradiography. The 32P-labeled proteins bands were excised from the PVDF blot and hydrolyzed in 6 M HCl for 24 h at 110°C. The acid-stable phospho-amino acids were separated by two-dimensional electrophoresis with the first dimension at pH 1.9 (750 V.h) in 7.8% acetic acid, 2.5% formic acid, followed by the second dimension at pH 3.5 (500V.h) in 5% acetic acid, 0.5% pyridine. After migration, radioactive molecules were detected by autoradiography. Authentic phosphoserine, phosphothreonine and phosphotyrosine were run in parallel and visualized using ninhydrin staining.

**Dot-blot immunoanalysis**

Zero or one μg of the His6-MDH non-phosphorylated or phosphorylated by 0.25 μg of PknD were spotted on the nitrocellulose membranes. The membranes were then incubated with 1 μg/ml of Flag-Rv1827 or Flag-Rv0020c for 16 h at 4°C with agitation. After washing, the Flag epitope was used to detect the bound Flag fusion proteins by chemiluminescence immunoanalysis using a mAb anti-Flag M2 (Sigma-Aldrich BVBA, Diegem, Belgium).

**MDH activity**

Malate dehydrogenase (EC 1.1.1.37) activity was assayed by spectrophotometry at 340 nm using an Uvikon 930 spectrophotometer. The assay was carried out in a 1 ml cuvette at room temperature in 100 mM glycine pH 9.0. The MDH activity was examined by measurement of the NADH consumption/production rate accompanying the reduction/oxidation of oxaloacetate/malate. An extinction coefficient of 6.2 x 10^3 M^-1cm^-1 was used to determine all kinetic parameters. To test the effect of phosphorylation on the activity of MDH, 0.15 μg of the MDH phosphorylated by 30 ng of PknA, PknD, PknE, or PknH was used for measurement in a 1 ml-cuvette containing 0.14 mM NADH and 0.8 mM oxaloacetate. A negative control was also run in parallel with the autophosphorylated kinase alone, in the absence of MDH.

**Immunoprecipitation**

*Mtb* cell pellets were resuspended in 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 5 mM EDTA, in the presence of the Complete protease inhibitor cocktail and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). Cells were lysed by sonication and membranes debris
were eliminated by centrifugation at 13,000 rpm for 10 min. The soluble extracts were incubated at 4°C during 4 h with protein-A sepharose beads pre-coupled with polyclonal antibodies against the MDH. After washing the beads, the antibody/antigen complexes were recovered by boiling for 5 min before SDS-PAGE electrophoresis.

Results

Identification of the M. bovis BCG PknD substrates by in vitro phosphorylation

To investigate the substrates of the PknD protein kinase, a soluble extract of M. bovis BCG was applied to a phenyl-Sepharose column CL-4B and the unbound pass-through material was separated by anion-exchange chromatography on a Resource-Q column. Samples from eluted fractions were tested in an in vitro phosphorylation assay using recombinant PknD protein and [γ-32P]ATP. As shown in Fig 1, several proteins in the various chromatographic fractions were phosphorylated by PknD. Five of these proteins were identified after sequencing using Edman degradation (Table 1). A phosphorylated protein present in lane 4 (chromatographic fractions 66–69, band 1) with an apparent MW around 37 kDa seemed to be the best substrate of the kinase. Its N-terminal sequence (SASPLKVAVT) was used to search by similarity with the BLAST (NCBI) program in the Sanger Mtb H37Rv database for the identity of the protein. The peptide fully matched the deduced N-terminal amino acid sequence of the Rv1240 ORF encoding the NAD+-dependent malate dehydrogenase (MDH). Further studies were thus performed on this enzyme, especially on its regulation by PknD.

In vitro phosphorylation of the recombinant MDH by PknD on threonine residue

The Mtb Rv1240 gene was cloned in frame into the expression vector pRSETc to produce an N-terminal His6-tagged recombinant protein. The protein expressed in a soluble form was purified to homogeneity with the predicted molecular mass of approximately 37 kDa (Fig 2). As shown in Fig 3A, the MDH was phosphorylated by PknD, but could not be autophosphorylated, confirming that MDH possessed no autokinase activity and that the observed phosphorylation of MDH was indeed mediated by PknD. Kinetic analysis of the phosphorylation showed that the phosphorylation occurred rapidly, reaching about 50% of its maximal rate within 4 min. After acid hydrolysis of the phosphorylated protein, the free amino acids were analyzed by two-dimensional electrophoresis. Comparison with phosphorylated standards showed that the phosphorylation had occurred on threonine residues (Fig 3B).

The MDH could be phosphorylated in vitro by other Mtb Ser/Thr protein kinases

Protein kinases exert their influence by recognizing a specific set of protein substrates. Mtb consists in the largest set of phosphorylated serines, threonines and tyrosines in bacteria (over 300 phosphoproteins) and the 11 Mtb STPKs have often been reported to phosphorylate common substrates [13, 14]. In order to find out the specificity of the MDH phosphorylation, nine other Mtb STPKs (PknA, PknB, PknE, PknF, PknG, PknH, PknJ, PknK and PknL) were used for in vitro phosphorylation assay. The full length of these kinases were all expressed in E. coli [22, 34] and fused at the N-terminus with a GST peptide. Their kinase activities were confirmed using the myelin basic protein (MBP) as a positive control (Fig 4). As shown in Fig 4, PknA, PknD, PknE, PknG, PknH and PknJ phosphorylated the (His)6-MDH.
PknD phosphorylated functional dimeric Mtb MDH

In most cases, eukaryotic MDHs are dimeric [35]. However, in some prokaryotes, the MDH can be tetrameric in its native state [36]. The functional relevance of the two MDH forms is not clear but the dimeric structure of the E. coli MDH is critical for its enzyme activity [37]. The active form of the Mtb MDH was a dimer as revealed by a native gel electrophoresis stained through a reaction catalyzed by the MDH activity (Fig 5A). This result suggested that the fusion of the His<sub>6</sub>-tag at the N-terminus of the MDH did not inhibit the formation of a dimer by MDH. As shown in Fig 5B, PknD phosphorylated the dimeric MDH in vitro.
Regulation of the MDH activity by PknD

The activity of the purified MDH enzyme was assayed using either oxaloacetate or malate as substrates. Table 2 summarized the values of the steady-state parameters. The $k_{cat}/K_m$ values for oxaloacetate and malate were about 17.40 and 0.29 respectively. The in vitro conditions to assay the activity of recombinant MDH favored the reduction of oxaloacetate coupled to the oxidation of NADH. After phosphorylation by PknD, the velocity of the NADH oxidation measured between 15 and 60 seconds decreased from $-0.509 \pm 0.025$/min to $-0.297 \pm 0.058$/min ($n = 3; P < 0.01$). The presence of the autophosphorylated PknD had no effect by itself on the oxidation of NADH (Fig 6). This indicated that the MDH phosphorylation by the PknD inhibited its activity (40% inhibition). In 2 other experiments, the activity of MDH was also inhibited after phosphorylation by 3 other kinases (42 and 53% inhibition for PknF; 23 and 32% inhibition for PknH; 27 and 38% inhibition for PknA). These results confirmed that the phosphorylation of MDH by STPKs regulated (inhibited) its activity.

The PknD phosphorylated MDH can bind to Rv1827 and Rv0020c, two proteins containing a FHA domain

The FHA domain recognizes phosphothreonine on proteins [25]. Five Mtb proteins have been found to contain at least one FHA domain [38]. Two of the corresponding genes Rv1827 and

Table 1. Determination of the major proteins phosphorylated by PknD in a homogenate from M. bovis.

| Band | Sequence | ID     | Database results                        | N° AA in the sequence | Site phosphorylation |
|------|----------|--------|-----------------------------------------|-----------------------|----------------------|
| 1    | SASPLKVAVT | Rv1240 | Malate dehydrogenase                    | 2–11                  | 6 Ser, 3 Thr        |
| 2    | AEVLLVEHAEG | Rv3038C | Electron transfer flavoprotein alpha subunit | 2–13                  | 5 Ser, 6 Thr        |
| 3    | TLNLXVDVL(T) | Rv3368C | Hypothetical protein Nitroreductase family | 2–13                  | 4 Ser, 3 Thr        |
| 4    | AEYTPLDLWDEY | Rv3846 | Superoxide dismutase chain A/B          | 2–13                  | 2 Thr               |
| 5    | ALPQLTDEGRAAALE | Rv1388 | MihF: mycobacterial integration host factor | 87–101                | 2 Ser, 2 Thr        |

Various fractions of the chromatography of a homogenate from M. bovis were incubated with PknD and the phosphorylated proteins were separated by SDS-PAGE. Five bands (numbered on the gels) were extracted and sequenced using the Edman degradation. They were identified by comparison with the genome of the H37Rv strain.

doi:10.1371/journal.pone.0123327.t001

Fig 2. SDS-PAGE of the His$_6$-MDH stained by Coomassie-Blue R-250. His$_6$-MDH was overproduced as a soluble protein and purified on a Ni$^{2+}$-immobilized HiTrap column. Lanes 1 (30 μg) and 2 (30 μg) show E. coli soluble extract before and after its loading on the HiTrap column, respectively. Lane 3 (1.5 μg) shows the purified His$_6$-MDH. The purified His$_6$-MDH was also observed by Western blot using a mAb anti-Xpress against His$_6$-tag epitope (lane 4).

doi:10.1371/journal.pone.0123327.g002
Rv0020c, have been cloned in frame with the flag epitope sequence in order to produce tagged proteins. The binding between these proteins and the His6-MDH was evaluated by dot-blot analysis. Phosphorylated and non-phosphorylated His6-MDH and controls were spotted on nitrocellulose membranes, which were then incubated with either flag-Rv1827 or flag-Rv0020c proteins. After washing, bound flag-Rv1827 and flag-Rv0020c were detected using an anti-flag monoclonal antibody. As shown in Fig 7, only the phosphorylated His6-MDH could bind to the Rv1827 and Rv0020c proteins. This result suggested that the association between His6-

Fig 3. In vitro phosphorylation of the MDH by the GST-PknD. A: Incubation with [γ-33P]ATP of the PknD alone (lane 1), the MDH alone (lane 2), the MDH with the GST-PknD for 30 sec (lane 3), 1 min (lane 4), 2 min (lane 5), 4 min (lane 6), 6 min (lane 7), 8 min (lane 8), 10 min (lane 9) and 15 min (lane 10). Proteins were separated by SDS-PAGE and radioactive bands were revealed by autoradiography. B: Phosphoamino acid content of the His6-MDH. The MDH was labeled with [γ-32P]ATP, analyzed by SDS-PAGE, transferred onto a PVDF membrane, excised, hydrolyzed in acid and separated by 2D electrophoresis on a TLC plate. Radioactive molecules were detected by autoradiography, whereas phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) as standard controls were run in parallel and visualized by ninhydrin staining. Phosphorylated FPΔIL-11 was used as a phosphoserine positive control and phosphorylated PknD as phosphoserine and phosphothreonine control.

Fig 4. In vitro phosphorylation of the MDH by other M. tuberculosis STPKs. Fifty nM of PknA, PknB, PknE, PknF, PknG, PknH, PknJ, PknK or PknL were incubated with [γ-32P]ATP in the absence or in the presence of MDH or not, respectively, for 30 min at 37°C. After phosphorylation, proteins were separated by SDS-PAGE, and radioactive bands were revealed by autoradiography.
MDH and Rv1827 or Rv0020c was not secondary to non-specific interactions but involved the phosphothreonine residue(s) on the phosphorylated MDH.

**Phosphorylation of the Mtb MDH during bacterial culture**

In order to determine whether the MDH was phosphorylated by the PknD within the bacteria, we analyzed by immunoprecipitation the MDH phosphorylation in the *Mtb* H37Rv wild-type

**Table 2. Kinetic parameters of the purified recombinant Mtb MDH.**

| Substrate/cofactor | $K_m$ (μM) | $k_{cat}$ (sec$^{-1}$) | $k_{cat} / K_m$ (μM$^{-1}$ sec$^{-1}$) |
|--------------------|------------|------------------------|--------------------------------------|
| Malate             | 1040       | 302                    | 0.29                                 |
| NAD$^+$            | 130        | 320                    | 2.46                                 |
| NADH               | 80         | 3330                   | 41.60                                |
| Oxaloacetate       | 140        | 2437                   | 17.40                                |

doi:10.1371/journal.pone.0123327.t002
and in the *Mtb* PknD-deficient (KO) mutant strains [30]. In normal culture condition, the MDH was always (early, middle and late exponential phases) expressed by both strains. Phosphorylated MDH was detected in the WT strain and in the PknD-KO mutant strain in middle and late exponential phases (Fig 8A). In agreement with *in vitro* experiments (Fig 4) this suggested that, *in vivo*, in normal conditions, the MDH could be phosphorylated by kinase(s) other than PknD. At the opposite, in phosphate deficient culture, the phosphorylation of MDH could be detected at the late exponential phase in the WT strain but not in the PknD-KO mutant (Fig 8A) suggesting that PknD had a more specific role in phosphorylating MDH than other kinases. When WT and PknD-KO *Mtb* were cultured in Dubos medium without oxygen, the MDH phosphorylation was detected in both strains at the middle exponential phase (OD_{600} = 0.6) (Fig 8B), implying that in hypoxia, the phosphorylation could be carried out by other STPK(s).

![Effect of PknD on MDH activity](image)

**Fig 6. Inhibition of the *in vitro* MDH activity by phosphorylation.** The MDH activity was assayed by measuring the decrease in NADH absorbance at 340 nm. The assay was carried out before (●) or after (▪) the phosphorylation of MDH by the PknD at a room temperature (20°C), or in the absence of MDH but in the presence of PknD (▲). Results are the means ± s.e.m. of *n* experiments performed in triplicates.
In preliminary experiments of this work we observed that PknD phosphorylated numerous proteins in *M. bovis*. Five of them were identified by N-terminal amino acids sequencing. In an extensive analysis of the phosphoproteome of mycobacteria, Prisic et al. (2010) reported that malate dehydrogenase (Rv 1240), an enzyme contributing to the TCA and the glyoxylate cycles and the mycobacterial integration host factor MihF (Rv 1388), the third most abundant protein of mycobacteria [39], were phosphorylated by STPKs [14]. The 3 other substrates of PknD, the iron factored superoxide dismutase A (Rv3846) essential for the survival of bacteria after intracellular infection [40], a classical nitroreductase (Rv3368C) and the alpha subunit of an electron transfer flavoprotein (Rv3028C), a protein overexpressed by INH-resistant strains and in response to osmotic stress [41], had not yet been reported as substrates of the STPKs. Our results further confirm the major contribution of these kinases, in particular PknD, as sensors/regulators of mycobacteria.

Among the five identified proteins, MDH was apparently the best substrate of PknD and it was phosphorylated both in vitro and in bacterial culture. Interestingly, Prisic et al. [14] identified more than 500 phosphorylation events occurring on 301 proteins in mycobacteria. Their data not only showed the MDH was phosphorylated by STPKs but mass spectrometry analysis also revealed that 3 residues could be phosphorylated. However, they did not identify whether serine or threonine were the phosphorylated amino acids. PknD is not only a STPK but also a tyrosine kinase [42] and we established that the phosphorylation of MDH by PknD occurred on threonine residues. We also showed that PknD phosphorylated the dimeric form of the enzyme. In vitro, 5 other STPKs (PknA, PknE, PknG, PknH and PknI) phosphorylated MDH and four other STPKs (PknB, PknF, PknK and PknL) were ineffective. These results were confirmed by experiments using PknD-KO mycobacteria. The phosphorylation of the MDH was increased when bacteria (both WT and PknD-KO strains) reached the growth stationary phase confirming that kinases other than PknD could phosphorylate MDH. These results are reminiscent of results reported in 2006 by Perez et al. [15]. Comparing the proteins phosphorylated in WT strain and in the KOD5 strain, a PknD mutant strain, they observed that the phosphorylation of several proteins with a MW around 37 KDa and different isoelectric pH, was weaker in the KOD5 strain than in the WT strain. This is consistent with the phosphorylation of a
37 kDa protein (MDH?) mostly by PknD but also by other kinases on several phosphorylation sites. Hypoxic conditions did not affect the phosphorylation of MDH in the presence or in the absence of PknD. In phosphate-poor medium, the phosphorylation of MDH was detected in WT mycobacteria but not in the PknD-KO strain. The phosphorylation by the PknD could
constitute a key step under phosphate-poor conditions, further emphasizing the role of this kinase as a sensor protein in phosphate signaling [43–46].

The phosphorylation of MDH had 2 major consequences for the enzyme. First it inhibited its in vitro activity. Considering that the assays were performed at saturating concentrations of substrates, the phosphorylation of the enzyme probably affected the maximal velocity of the enzyme rather than its affinity for its substrates. Second it also promoted its interaction with other proteins. Rv0020c and Rv1827, two of the five Mtb FHA-containing proteins. This suggested that the interactions between these proteins were phosphothreonine-dependent, in agreement with the FHA domain characteristics. The Rv1827/GarA which is regulated by phosphorylation via STPK [28], has also a phospho-independent domain capable of interacting with three non-phosphorylated metabolic enzymes (α-ketoglutarate decarboxylase, NAD+-dependent glutamate dehydrogenase and the α-subunit of glutamate synthetase) inhibiting their contribution to the TCA cycle and the synthesis of glutamate [27, 47]. The interaction of MDH with the Rv0020c and the Rv1827/GarA proteins might participate to the regulation of the intermediary metabolism by FHA-containing proteins. It should be noted that the phosphorylation of MDH inhibited its activity in vitro, in the absence of FHA-containing proteins, demonstrating that the interaction between these proteins and MDH was not involved in the short-term regulation of MDH by phosphorylation. This interaction might play an intermediate role in targeting the phosphorylated MDH to proteolysis [48].

Within a granuloma, mycobacteria are exposed to hypoxic, acidic conditions and also to low concentrations of nutrients and high concentrations of reactive oxygen and nitrogen intermediates. In phagolysosomes mycobacteria are also exposed to a phosphate-depleted medium [49]. The phosphorylation of the MDH by STPKs when Mtb entered the stationary phase, when Mtb suffered from lack of phosphate or when Mtb grew under oxygen deprivation and the subsequent inhibition of the catalytic activity of the enzyme, suggest that this regulation might be one of the mechanisms of environmental adaptation allowing Mtb to survive under poor nutrients and/or hypoxic conditions and entering into a nonreplicating or dormant form [32, 50]. Our results confirm that PknD contributes to this regulation. The extracellular C-terminal domain of this kinase is a sensor domain which forms a funnel-shaped propeller structure containing six blades arranged cyclically around a central pore [51]. A flexible domain connects this extracellular domain to the transmembrane domains and after activation, mediates signaling through activation of the intracellular domain with the kinase activity. Recent data by Baer et al. showed that PknD was cross-phosphorylated by PknB and by a cascade successively involving PknH and PknE [52]. Considering that different signaling pathways converge to PknD and that this kinase autophosphorylates and autocatalytically increases its activity [53], PknD might thus be a major step in the regulation of the metabolic activity of the mycobacteria in the granuloma.

Acknowledgments

We thank Dr. Anil Koul for the gift of the recombinant serine/threonine protein kinases from Mtb.

Author Contributions

Conceived and designed the experiments: XMW PL. Performed the experiments: XMW KS. Analyzed the data: XMW KS VF JPD PL. Contributed reagents/materials/analysis tools: PP MK. Wrote the paper: XMW VF JPD PL.
References

1. Stewart GR, Robertson BD, Young DB (2007) Tuberculosis: a problem with persistence. Nat Rev Microbiol 1: 97–105.

2. Via LE, Lin PL, Ray SM, Carrillo J, Allen SS, Eum SY, et al. (2008) Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. Infect Immun 76:2339–2340. doi: 10.1128/IAI.01515-07 PMID: 18347040

3. Bloom BR, McKinney JD (1999) The death and resurrection of tuberculosis. Nat Med 5: 872–874. PMID: 10426305

4. Smith CV, Sharma V, Sacchettini JC (2004) TB drug discovery: addressing issues of persistence and resistance. Tuberculosis 84: 45–55. PMID:14670345

5. Berney M, Cook GM (2010) Unique flexibility in energy metabolism allows mycobacteria to combat starvation and hypoxia. PlosOne 5: e8614. doi: 10.1371/journal.pone.0008614 PMID: 20062806

6. Bishai W (2000) Lipid lunch for persistent pathogen. Nature 406: 683–685. PMID:10963578

7. Daniel J, Deb C, Dubey VS, Sirakova TD, Abomoelak B, Morbidoni HR, et al. (2004) Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in Mycobacterium tuberculosis as it goes into a dormancy-like state in culture. J Bacteriol 186: 5017–5030. PMID:15262939

8. Watanabe S, Zimmermann M, Goodwin MB, Sauer U, Boshoff HI (2011) Fumarate reductase activity maintains an energized membrane in anaerobic Mycobacterium tuberculosis. Plos Pathog 7:e1002287. doi: 10.1371/journal.ppat.1002287 PMID: 21998585

9. Shi L, Sohaskey CD, Pfeiffer C, Parks M, McFadden J, et al. (2004) Induction of a Dormancy-like state in culture. J Bacteriol 186: 5017–5030. PMID:15262939

10. Beste DJV, Bonde B, Hawkins N, Ward JL, Beale MH, Noack S, et al. (2011)13C Metabolic flux analysis identifies an unusual route for pyruvate dissimilation in mycobacteria which requires isocitrate lyase and carbon dioxide fixation. Plos Pathog 7:e1002091. doi:10.1371/journal.ppat.1002091 PMID: 21814509

11. Wehenkel A, Bellinzoni M, Grana M, Ranganath P, Villarino A, Fernandez P, et al. (2008) Mycobacterium Ser/Thr protein kinases and phosphatases: physiological roles and therapeutic potential. Biochim Biophys Acta 1784: 193–202. PMID: 17869195

12. Chao J, Wong D, Zheng X, Poirier V, Bach H, Hmama Z, et al. (2010) Protein kinase and phosphatase signaling in Mycobacterium tuberculosis physiology and pathogenesis. Biochim Biophys Acta 1804: 620–627. doi: 10.1016/j.bbabap.2009.09.006 PMID: 19766738

13. Cousin C, Derouiche A, Shi L, Pagot Y, Poncet S, Mijakovic I (2013) Protein-serine/threonine/tyrosine kinases in bacterial signal transduction and regulation. FEMS Microbiol Lett 346: 11–19. doi: 10.1111/j.1574-6968.2013.2181509

14. Prisic S, Dankwa S, Schwartz D, Chou MF, Locasale JW, Kang CM, et al. (2010) Extensive phosphorylation with overlapping specificity by Mycobacterium tuberculosis serine/threonine protein kinases. Proc Nat Acad Sci U S A 107: 7521–7526. doi: 10.1073/pnas.0914821107 PMID: 20368441

15. Perez J, Garcia R, Bach H, de Waard JH, Jacobs WR Jr., Av-Gay Y, et al. (2006) Mycobacterium tuberculosis transporter MmpL7 is a potential substrate for kinase PknD. Biochem Biophys Res Commun 348: 6–12. PMID: 16879601

16. Greenstein AE, MacGurn JA, Baer CE, Falick AM, Cox JS, Alber T (2007) M. tuberculosis Ser/Thr protein kinases phosphorylate an anti-anti-sigma factor homolog. PLoS Pathog 3: 475–483.

17. Park ST, Kang CM, Husson RN (2008) Regulation of the SigH stress response regulon by an essential serine/threonine protein kinase. Proc Natl Acad Sci U S A 105: 13105–13110. doi: 10.1073/pnas.0801134105 PMID: 18728196

18. Hatzios SK, Baer CE, Rusted TR, Siegrist MS, Pang JM, Ortega C, et al. (2013) Osmosensory signaling in Mycobacterium tuberculosis mediated by a eukaryotic-like Ser/Thr protein kinase. Proc Natl Acad Sci U S A 110: E5069–E5077. doi: 10.1073/pnas.1321205110 PMID: 24309377

19. Kumar P, Kumar D, Parikh A, Rananaware D, Gupta M, Singh Y, et al. (2009) The Mycobacterium tuberculosis protein kinase K modulates activation of transcription from the promoter of mycobacterial monoxygenase operon through phosphorylation of the transcriptional regulator VirS. J Biol Chem 284: 11090–11099. doi: 10.1074/jbc.M807052200 PMID: 19251699

20. Lee JJ, Kan CM, Lee JH, Park KS, Jeon JH, Lee SH (2014) Phosphorylation-dependent interaction between a serine/threonine kinase PknA and a putative cell division protein Wag31 in Mycobacterium tuberculosis. New Microbiol 37: 525–533. PMID: 25387290
21. Jayakumar D, Jacobs WR Jr, Narayanan S (2008) Protein kinase E of Mycobacterium tuberculosis has a role in the nitric oxide stress response and apoptosis in a human macrophage model of infection. Cell Microbiol 10: 365–374. PMID: 17892498

22. Walburger A, Koul A, Ferrari G, Nguyen L, Prescianotto-Baschong C, Huygen K, et al. (2004) Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. Science 304: 1800–1804. PMID: 15155913

23. Grundner C, Gay LM, Alber T (2005) Mycobacterium tuberculosis serine/threonine kinases PknB, PknD, PknE, and PknF phosphorylate multiple FHA domains. Prot Sci 14: 1918–1921.

24. Pennell S, Westcott S, Ortiz-Lombardía M, Patel D, Li J, Nott TJ, et al. (2010) Structural and functional analysis of phosphothreonine-dependent FHA domain interactions. Structure 18: 1587–1595. doi: 10.1016/j.str.2010.09.014 PMID: 21134638

25. Mollé V, Soulaut D, Jault JM, Grangeasse C, Cozzone AJ, Prost JF (2004) Two FHA domains on an ABC transporter, Rv1747, mediate its phosphorylation by PknF, a Ser/Thr protein kinase from Mycobacterium tuberculosis. FEMS Microbiol Lett 234: 215–223. PMID: 15135525

26. Durocher D, Jackson SP (2002) The FHA domain. FEBS Lett 513: 58–66. PMID: 11911881

27. Ventura M, Rieck B, Boldrin F, Degiacomi G, Bellinzoni M, Barilone N, et al. (2013). GarA is an essential regulator of metabolism in Mycobacterium tuberculosis. Mol Microbiol 90: 356–366. doi: 10.1111/mmi.12368 PMID: 23962235

28. England P, Wehenkel A, Martins S, Hoos S, André-Leroux G, Villarino, et al. (2009) The FHA-containing protein GarA acts as a phosphorylation-dependent molecular switch in mycobacterial signaling. FEBS Lett 583: 301–307. doi: 10.1016/j.febslet.2008.12.036 PMID: 19114043

29. Roumestand C, Leiba J, Galophe N, Margeat E, Padilla A, Bessin Y, et al. (2011) Structural insight into the Mycobacterium tuberculosis Rv0020c protein and its interaction with the PknB kinase. Structure 19: 1525–1534. doi: 10.1016/j.str.2011.07.011 PMID: 22000520

30. Vanzemhergh F, Peirs P, Leffeve P, Celio N, Mathys V, Content J, et al. (2010) Effect of PstS sub-units or PknD deficiency on the survival of Mycobacterium tuberculosis. Tuberculosis 90: 338–345. doi: 10.1016/j.tube.2010.09.004 PMID: 20933472

31. Wayne LG, Lin KY (1982) Glyoxylate metabolism and adaptation of Mycobacterium tuberculosis to survival under anaerobic conditions. Infect Immun 37: 1042–1049. PMID: 6813266

32. Wayne LG, Soshaskey CD (2001) Nonreplicating persistence of Mycobacterium tuberculosis. Annu Rev Microbiol 55: 139–163. PMID: 11544532

33. Cannata JJ, Cazzullo JJ (1984) Glycosomal and mitochondrial malate dehydrogenases in epimastigotes of Trypanosoma cruzi. Mol Biochem Parasitol 11: 37–49. PMID: 6379451

34. Nguyen L, Walburger A, Houben E, Koul A, Muller S, Morbitzer M, et al. (2005) Role of protein kinase G in growth and glutamine metabolism of Mycobacterium bovis BCG. J Bacteriol 187: 5852–5856. PMID: 16077135

35. Birktoft JJ, Fu Z, Camahan GE, Rhodes G, Roderick SL, Banaszak LJ (1989) Comparison of the molecular structures of cytoplasmic and mitochondrial malate dehydrogenase. Biochem Soc Trans 17: 301–304. PMID: 2753208

36. Dahlu B, Saarinen M, Sauer UH, Eklund P, Johansson K, Karlsson A, et al. (2002) Structural basis for thermophilic protein stability: structures of thermophilic and mesophilic malate dehydrogenases. J Mol Biol. 318:707–721. PMID: 12054817

37. Breiten DR, Resnik E, Banaszak LJ (1994) Engineering the quaternary structure of an enzyme: construction and analysis of a monomeric form of malate dehydrogenase from Escherichia coli. Protein Sci. 3:2023–2032 PMID: 7703849

38. Cole ST, Brosch R, Parkinson J, Garnier T, Churcher C, Harris D (1998) Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393: 537–544. PMID: 9634230

39. Schubert OT, Mortensen J, Ludwig C, Röst HL, Rosenberger G, Arthur PK, et al (2013) The Mtb proteome library: a resource of assays to quantify the complete proteome of Mycobacterium tuberculosis. Cell Host Microbe 13: 597–602. doi: 10.1016/j.chom.2013.04.008 PMID: 23894311

40. Edwards KM, Cynamon MH, Hager CC, DeStefano MS, Tham KT, et al. (2001) Iron-cofactor superoxide dismutase inhibits host responses to Mycobacterium tuberculosis. Am J Respir Crit Care Med 164: 2213–2219. PMID: 11751190

41. Jiang X, Zhang W, Gao F, Huang Y, Lv C, Wang H (2006) Comparison of the proteome of isoniazid-resistant and susceptible strains of Mycobacterium tuberculosis. Microb Drug Resist 12: 231–238. PMID: 17227207

42. Kusebauch U, Ortega C, Ollodart A, Rogers SR, Sherman DR, Moritz RL, et al. (2014) Mycobacterium tuberculosis supports protein tyrosine phosphorylation. Proc Natl Acad Sci U S A 111: 9265–9270. doi: 10.1073/pnas.1323894111 PMID: 24927537
43. Av-Gay Y, Everett M (2000) The eukaryotic-like Ser/Thr protein kinases of Mycobacterium tuberculosis. Trends Microbiol 8: 238–244. PMID:10785641

44. Peirs P, Parmentier B, De Wit L, Content J (2000) The Mycobacterium bovis homologous protein of the Mycobacterium tuberculosis serine/threonine protein kinase Mbk (PknD) is truncated. FEMS Microbiol Lett 188: 135–139. PMID: 10913696

45. Peirs P, De Wit L, Braibant M, Huygen K, Content J (1997) A serine/threonine protein kinase from Mycobacterium tuberculosis. Eur J Biochem 244: 604–612. PMID: 9119030

46. Lefevre P, Braibant M, De Wit L, Kalai M, Roepers D, Grötzinger J, et al. (1997) Three different putative phosphate transport receptors are encoded by the Mycobacterium tuberculosis genome and are present at the surface of Mycobacterium bovis BCG. J Bacteriol 179: 2900–2906. PMID: 9139906

47. O’Hare HM, Duran R, Cervenansky C, Bellinzoni M, Wehenkel AM, Pritsch O, et al. (2008) Regulation of glutamate metabolism by protein kinases in mycobacteria. Mol Microbiol 70: 1408–1423. doi: 10.1111/j.1365-2958.2008.06489.x PMID: 19019160

48. Li J, Lee G, Van Doren SR, Walker JC (2000) The FHA domain mediates phosphoprotein interactions. J Cell Sci 113: 4143–4149. PMID: 11069759

49. Rengarajan J, Bloom BR, Rubin EJ (2005) Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. Proc Natl Acad Sci U S A 102: 8327–8332. PMID: 15928073

50. Rifat D, Bishai WR, Karakousis PC (2009) Phosphate depletion: a novel trigger for Mycobacterium tuberculosis persistence. J Infect Dis 200: 1126–1135. doi: 10.1086/605700 PMID: 19686042

51. Good MC, Greenstein AE, Young TA, Ng HL, Alber T (2004) Sensor domain of the Mycobacterium tuberculosis receptor Ser/Thr protein kinase, PknD, forms a highly symmetric beta propeller. J Mol Biol 339: 459–469. PMID: 15136047

52. Baer CE, Javarone AT, Alber T, Sassetti CM (2014) Biochemical and spatial coincidence in the provisional Ser/Thr protein kinase interaction network of Mycobacterium tuberculosis. J Biol Chem 289: 20422–2043. PMID: 24928517

53. Durán R, Villarino A, Bellinzoni M, Wehenkel A, Fernandez P, Boitel B, et al. (2005) Conserved autophosphorylation pattern in activation loops and juxtamembrane regions of Mycobacterium tuberculosis Ser/Thr protein kinases. Biochem Biophys Res Commun 333: 858–867. PMID: 15967413