Materials and Methods

Bacterial strains, plasmids, media and chemicals. E. coli strain BL21(DE3) was used as the host for protein expression. Plasmid pET28a (Novagen) – which carries the T7 promoter, an hexahistidine tag coding sequence and kanamycin resistance gene – was used as expression vector for *Mt*_PtpA and *Mt*_PtpB[14, 15]. Plasmid pET19b (Novagen) was used as expression vector for human PTP1B (37kDa, residues 1-321; provided by Dr. Tiago Brandão). Isopropyl-β-D-thiogalactopyranoside (IPTG), antibiotics and all the other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All products were of analytical or reagent grade.

Site-directed mutagenesis. Selected codons in the pET28a-*Mt*_PtpA recombinant plasmid previously obtained[14] were mutated by PCR in order to obtain the following amino acid substitutions in the *Mt*_PtpA: C11A, C16A and C53A (residue numbering is for *Mt*_PtpA). The QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene) was used to mutate the selected codons. The following primers were used: (i) to change Cys11 to Ala 5'- GTC ACA TTC GTT ***GCG*** ACG GGC -3', (ii) to change Cys16 to Ala 5'- ACG GGC AAC ATC ***GCA*** CGG TCG CCA ATG -3', (iii) to change Cys53 to Ala 5'- CAT GTA GGC AGT ***GCA*** GCA GAC GAG CGG GCG -3' (bold face italics denote the changed nucleotides). The recombinant plasmids were used to transform *E. coli* XL10-Gold ultracompetent cells. The mutations were verified by DNA sequencing.

Expression and purification of Ptps. In order to express the recombinant *Mt*_PtpA and *Mt*_PtpB, plasmid pET28a-*Mt*_PtpA and pET28a-*Mt*_PtpB were transformed into *E. coli* BL21(DE3). *E. coli* cells containing the recombinant plasmids were inoculated into

10 ml of LB broth containing 50 µg/ml kanamycin. Overnight cultures were transferred to 250 ml of fresh medium and were grown at 37 ºC until an OD value of 0.8 at 600 nm was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, cultures were further grown overnight at 15 ºC. Cells were harvested by centrifugation (6,000*g* for 15 min at 4 ºC) and re-suspended in cold lysis buffer (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 10 mM imidazole, 10% glycerol) containing 40 µg/ml of protease inhibitor (PMSF- phenylmethylsulfonyl fluoride, Sigma® Aldrich). The cells were disrupted by gentle sonication (7 cycles, 20s) on ice and centrifuged (13,000*g* for 20 min at 4 ºC).

*Mt*_PtpA and *Mt*_PtpB carrying the hexahistidine tag at the N-terminus were purified under native conditions using HisTrap HP columns (GE Healthcare Bio-Sciences) connected to an Äkta System (Amersham Biosciences). The hexahistidine tagged proteins were eluted in a 100-500 mM imidazole gradient. The purity of the protein preparations was assessed by SDS-PAGE in 16% acrylamide slab gels, under reducing conditions. Fractions containing purified recombinant protein were pooled and dialyzed at 4°C, in four steps, for 2 h against the elution buffer with decreasing concentrations of imidazole (150 mM, 75 mM, 35 mM and without imidazole). The last dialysis buffer contained 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM EDTA, 20% glycerol, 5 mM DTT. Then, proteins were concentrated by ultrafiltration with a 10 kDa pore membrane (Amicon Ultra-15 Millipore) and stored at -80°C for activity assays, or dialyzed against HEN buffer (250 mM Hepes pH 7.7, 1 mM EDTA, 0.1 mM neocuproine) for biotin switch assays. Protein concentration was determined using the method of Bradford, with bovine serum albumin as standard. *Mt*_PtpA mutant proteins were expressed and purified as described for wild-type *Mt*_PtpA. PTP1B was expressed and purified as described previously[15].

Enzyme assays. The activity of PTP1B, PtpB, PtpA WT and mutants was measured at 37 °C in 96-well microplates. Reactions were performed in a reaction buffer composed of 20 mM imidazole pH 7.0, 50 nM of enzyme and 20 mM *p*-nitrophenyl phosphate (*p*NPP) as substrate. The amount of *p*-nitrophenol (pNP) produced was followed for 10 min at 410 nm and its production was quantified using a *p*NP molar absorption coefficient of 2249.6 $M^{-1}cm^{-1}$ (experimentally determined for the above described conditions). Control reactions without enzymes were included to account for the spontaneous hydrolysis of pNPP.

For nitrosylation assays, S-nitrosoglutathione (GSNO) was used as a NO donor, with glutathione as a control. Enzymes were preincubated with 1 mM SNOG or GSH for 30 min at 25 °C in the dark and then added to the reaction mixture. Phosphate release was calculated as a percentage of the specific activity of the untreated samples. All assays were performed in triplicate.

Biotin switch assay for detection of S-nitrosylated proteins. The biotin switch technique was performed on purified proteins as described below. Protein concentration was adjusted to a maximum of 0.8 mg/ml. Samples were treated with 1 mM GSNO for 30 min at room temperature (RT) in the dark, and then were incubated for 30 min at 50°C with 4 volumes of blocking buffer (250 mM Hepes pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 2.5% (w/v) SDS, 27 mM MMTS). Samples were frequently vortexed for blocking non-nitrosylated free Cys residues. To differentiate between specific and non-specific nitrosylations, a set of samples was treated with 1 mM GSH instead of GSNO or with 10 mM DTT after GSNO treatment. Residual MMTS was removed by precipitation with 10 volumes of chilled (-20 ºC) acetone and the proteins were resuspended in HENS buffer (250 mM Hepes pH 7.7, 1 mM EDTA,

0.1 mM neocuproine, 1 % (w/v) SDS). S-nitrosylated residues were reduced with 1 mM ascorbate for 10 min and biotinylation was achieved by adding 2 mM biotin-HPDP (Pierce) and incubating at room temperature for 1 h. As a negative control, a set of GSNO-treated samples was incubated with DMSO instead of biotin. In order to detect biotinylated proteins by western blot, samples from the biotin switch assay were separated on 16% SDS–PAGE gels, transferred to PVDF membranes, blocked with non-fat dried milk, and incubated with 1/10,000 mouse antibiotin antibody (Sigma) for 1 h at RT. The membranes were then washed and incubated 1 h with secondary anti-mouse IgG conjugated with peroxidase. Amersham ECL chemiluminescent kit was used to detect the signals, and Amersham Hyperfilm ECL films to visualize the results.

Structural PtpA Cys53-NO schematic view. The PtpA–Cys53-NO structural representation was built using the PtpA crystal structure (PDB entry 1U2P) from *M. tuberculosis*. The NO ligand was introduced acquiring the atomic coordinates from the S-NO Cys215 in the human PTP1B structure (PDB entry 3EUO). The solvent accessibility was obtained using *Swiss-pdbviewer* 4.0.1 software (www.expasy.org/spdbv/) and the figures were prepared using *Pymol* software (2003, DeLano Scientific Ltd, Palo Alto, CA, USA).