SUPPORTING INFORMATION

Structure, biochemistry, and inhibition of essential 4′-phosphopantetheiny transferases from two species of *Mycobacteria*

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| Sfp | PptT |
|-----|------|
| **Std** | apo-ACP | apo-ACP |
| A | H | A | H |
| + | - | + | - |
| + | - | + | - |
| (+) CoA | apo/holo Sfp | apo/holo PptT |
| H | A | A | A |
Supplementary figure 11: Raw ITC data. Sfp is depicted on the left, and PptT on the right. Curves were calculated using the single binding site equation in the Origin software package (Microcal/GE Healthcare). Values of $n$ (ratio of ligand to protein), $K$ (Thermodynamic constant), $\Delta H$ (enthalpy), and $\Delta S$ (entropy) are displayed in tables inset into the titration graphs. (a) Sfp and PptT titrated with CoA after CIP treatment. (b) Sfp and PptT titrated with Rhodamine-CoA. Thermodynamic parameters could not be calculated for PptT with Rhodamine-CoA.
|                      | PptT (SeMet)                  | MuPPT                  |
|----------------------|-------------------------------|------------------------|
| Resolution range, Å  | 41.22 - 1.59 (4.87 - 1.59)   | 37.24 - 1.65 (1.709 - 1.65) |
| Space group          | C 2 2 21                      | P 21 21 21             |
| a, b, c; Å           | 99.86, 121.51, 48.78          | 55.02, 59.75, 74.48    |
| α, β, γ; (°)         | 90, 90, 90                    | 90, 90, 90             |
| Total reflections    | 723703 (49707)                | 403854 (35853)         |
| Unique reflections   | 44112 (4201)                  | 25868 (2879)           |
| Multiplicity         | 9.1 (7.2)                     | 5.6 (2.7)              |
| Completeness (%)     | 99.58 (97.6)                  | 85.5 (66.7)            |
| Mean I/σ(I)          | 8.5 (1.1)                     | 9.1 (2.3)              |
| R-merge              | 0.171 (2.134)                 | 0.15 (0.388)           |
| R-meas               | 0.182 (2.318)                 | 0.163 (0.470)          |
| R-work               | 0.1708                        | 0.1599                 |
| R-free               | 0.2026                        | 0.2085                 |
| Number of non-hydrogen atoms | 2114 | 2039 |
| macromolecules       | 1779                          | 1761                   |
| ligands              | 58                            | 49                     |
| water                | 277                           | 229                    |
| Protein residues     | 228                           | 228                    |
| RMS(bonds)           | 0.011                         | 0.031                  |
| RMS(angles)          | 1.381                         | 1.45                   |
| Ramachandran favored (%) | 99.57 | 99.12 |
| Ramachandran outliers (%) | 0     | 0      |
| Clashscore           | 2.74                          | 2.50                   |

Statistics for the highest-resolution shell are shown in parentheses.

Supplementary Table 1: crystallographic parameters of PptT and MuPPT structures.
| PPTase | $K_m$ CoA (µM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (µM$^{-1}$min$^{-1}$) |
|--------|----------------|------------------------|-----------------------------------|
| PptT   | 1.600±0.248    | 0.42 ± 0.012           | 0.26 ± 0.05                       |
| MuPPT  | 2.637±0.502    | 0.66 ± 0.026           | 0.25 ± 0.06                       |
| Sfp    | 11.69±2.56     | 0.18 ± 0.012           | 0.020 ± 0.004                     |

Supplementary table 2: Relative kinetic parameters obtained from the BpsA assay for PptT, MuPPT, and Sfp at 50 nM PPTase. $K_m$ is measured with respect to CoA as the variable substrate.
| Primer name | Sequence |
|-------------|----------|
| MBP_F1      | AAAAATCTAGACTCCTTCTTTAAAGTTAAATGAAAATCGAAGAAGGTTAAA |
|             | CTGGTAATC |
| MBP_R1      | CGTGGAAACCAGTCCGCTGCTCCCGAGGTGTTG |
|             | AAAAATCTAGATTATACTTTAAGAAGGAGTATAATGAAAATCGAAGAA |
| MBP_F2      | GG |
| MBP_R2      | AAAAACATATGGGATCCTCGTGGAACCAGTCCGCTGCTCCCGAGG |
| E157Q_F     | TTGTGCAAACAAGGCAACTTACAAAGCATG |
| E157Q_R     | TAAGTTGCTGTTTTTGCAACAAAAACAGGATA |
| E116Q_F     | TATCGATGCTCAACCTCATGATGTCTGCC |
| E116Q_R     | TCATGAGGTTGAGCATCGATACCTACGCGT |
| D114N_F     | CGTAGGTATCAATGCTGAACCTCATGATGT |
| D114N_R     | GGTTCAGCATTGATACCTACGATCATGAG |
| R48A_F      | CGTTGCAAAAAGCCCGTAACGAATTATTAC |
| R48A_R      | AATTCGTTACGGGCTTTTGCAACGCTACGA |
| R56A_F      | GAATTCATTACCGTGCTGTCATTCGCG |
| R56A_R      | CGCGCAATGAGCCAGGTTAAATATTCGTT |

Supplementary table 3: primers used for cloning shown in the 5' to 3' direction.
Supplementary table 4: Structures of inhibitors tested for inhibitory activity measured by IC$_{50}$.

| Compound                          | MAS  | VibB | Compound                          | MAS  | VibB |
|-----------------------------------|------|------|-----------------------------------|------|------|
| CoA                               | 1.1 ± 0.1 | 4.7 ± 0.4 | 6-nitroso-benzopyrone           | 24 ± 2 | 17 ± 2  |
| 3',5'-phosphoadenylyl phosphate   | 1.6 ± 0.2 | 0.78 ± 0.20 | PD 404,182                   | 19 ± 1 | 7.1 ± 0.4 |
| 2'-deoxy-3',5'-phosphoadenylyl phosphate | 7.4 ± 0.4 | 8.5 ± 1.0 | Guanidinyl-naltrindole difluoracetate | 12 ± 1 | 19 ± 1  |
| Benserazide HCl                   | NC   | NC   | Sanguinarine Cl                   | 4.9 ± 0.2 | 22 ± 2  |
| Mitoxantrone 2HCl                 | Inactive | Inactive | Calmidazolium Cl                 | 4.9 ± 0.4 | 2.0 ± 0.2 |
| SCH-202676                        | 0.5*  | 0.8*  | (-)-ephrine hemisulfate          | Inactive | Inactive |
| Bay 11-7085                       | 30 ± 4 | NC   |                                  |       |      |
**Methods**

**Cloning and purification MBP-PptT**

PptT was codon optimized for *E. coli* and synthesized by DNA 2.0 in pJ201, and subcloned into pET24b (Novagen) using restriction sites NdeI and XhoI. The MBP sequence was duplicated from the pMAL-c2 (New England Biolabs) plasmid with primers MBP_F1 and MBP_R1 to begin addition of the thrombin cleavage site followed by PCR with MBP_F2 and MBP_R2 to finish creation of the thrombin cleavage site, as well as optimize the RBS from the first fragment and add the NdeI site on the 3’ end in preparation for vector insertion. Following double digestion with XbaI/NdeI and treatment with shrimp alkaline phosphatase (NEB), the desired MBP DNA was ligated into the parent pET24b/PptT vector.

This MBP construct was transformed into BL-21 DE3 chemically competent cells and grown at 37°C to an O.D. of 0.8 in LB media supplemented with 2g L⁻¹ glucose, induced with 1 mM IPTG, and grown overnight at 18°C. Cells were pelleted and lysed into 50 mM Tris pH 8.0 and 500 mM NaCl. Lysate was passed over amylose resin and washed with lysis buffer. MBP-PptT was eluted from the column with lysis buffer plus 1 mM DTT and 25 mM maltose. The elutant was concentrated and used without further purification.

**Cloning, Expression, and Purification of PPTases and carrier protein targets**

MuPPT was codon optimized for *E. coli* and synthesized by Genscript. Both PptT and MuPPT were subcloned into pET24b using the restriction sites NdeI and XhoI. BL-21 DE3 chemically competent cells were transformed with the pET24b plasmid containing PptT or MuPPT. Cells were grown to an O.D. of 0.8 in LB media at 37°C, induced with 1 mM IPTG, and grown overnight at 16°C. The cells were lysed into a lysis buffer consisting of 50 mM MES pH 6.2, 500 mM NaCl, 1 mM CaCl₂, 10% (v/v) glycerol, and 20 mM imidazole. PptT was purified over Ni-NTA (and eluted with Lysis Buffer containing 250 mM imidazole. The elutant was concentrated and the imidazole was removed using a PD-10 desalting column (GE healthcare) equilibrated with 50 mM MES pH 5.8, 500 mM NaCl, 1 mM
CaCl$_2$, and 10% (v/v) glycerol. MuPPT and all PptT mutants were purified as described for PptT. Se-Met PptT was grown in M9 minimal media supplemented by an amino acid cocktail that included L-Selenomethionine (Sigma). Purification was carried out in the same manner as described above.

Protein destined for crystallization was then further purified on a Sephadex S200 (GE Healthcare) size exclusion column equilibrated with crystallization buffer consisting of 20 mM MES pH 5.8, 100 mM NaCl, and 5 mM MgCl$_2$, and was concentrated to 8 mg mL$^{-1}$ measured by Bradford method. 5 mM DTT and 1 mM CoA were added prior to crystallization.

Sfp$^8$, VibB$^9$, AcpP$^{10}$, and MAS$^{10}$ were produced and purified as previously described.

Primers for MBP fusion construction and PptT mutants are listed in table S3.

**Crystallization techniques**

Using hanging drop vapor diffusion, medium sized plate-like crystals of PptT formed over a period 6 days in 100 mM Sodium acetate pH 4.5, 200 mM LiSO$_4$, and 30% (w/v) PEG 8000, which was discovered from Wizard Screen I (Emerald Biosystems). Diffraction quality crystals were obtained when drops consisting of 2 µL protein and 1 µL buffer solution were hung over an empty reservoir. Crystals were frozen in a cryo solution consisting of crystallization buffer plus 15% (v/v) Ethylene Glycol. Large rod-like crystals of MuPPT formed over a period of 1-2 months in drops consisting of 2 µL protein and 1 µL 2M LiCl, 32% (w/v) PEG 8000, pH 5.5. Crystals were frozen in a cryo solution consisting of mother liquor plus 15% (v/v) Ethylene Glycol.

**Data Collection and processing**

Data was collected on beamlines 8.2.1 and 8.2.2 at the Advanced Light Source (Berkeley, CA, USA). Raw data was indexed with mosflm$^{11}$ and scaled with scala using the CCP4 suite.$^{12}$ Initial models using phasing data from Se-Met PptT and all refinement was performed with Coot$^{13}$ and Phenix.$^{14}$ PptT was phased using single wavelength anomalous diffraction data from Se-Met labeled protein. MuPPT was
phased using the previously solved PptT structure. All figures depicting crystal structures were prepared using PyMol.  

Gel based analysis of labeling of MAS, VibB, and *E. coli* ACP were performed as previously described.  

**Removal of pre-bound CoA from PPTases**

10 mg of Sfp was treated with 100 U CIP (Worthington Biochemical) in 5 mL 50 mM TrisCl pH 8, 250 mM NaCl, 10% (v/v) glycerol, 10 mM MgCl₂, along with a sufficient quantity of nickel resin for binding. Incubation of the mixture proceeded with rocking at room temperature for 2 hours. Nickel resin was washed with 50 mM MES pH 6.2, 500 mM NaCl, 10% (v/v) glycerol, 1 mM CaCl₂ and then eluted with 300 mM imidazole in MES buffer. Eluted CIP-treated Sfp was buffer exchanged with a centrifugal filter prior to use. PptT was prepared in the same manner as Sfp, except for a pre-binding of PptT to Ni-NTA resin for 1 hour on ice, prior to buffer exchanging to the Tris CIP reaction buffer for CIP treatment. CIP-treated, desalted PPTase at 20 µM was combined with *apo* E. coli ACP at 100 µM with 50 mM HEPES pH 7.6 and 10 mM MgCl₂ at 37°C overnight without coenzyme A to qualitatively gauge the removal of pre-bound coenzyme A from PPTases. Additional controls including untreated PPTase demonstrate lower conversion from *apo-* to *holo-*CP upon CIP treatment, as well as demonstrate retention of activity with the re-addition of coenzyme A. Samples were run on 20% Urea-PAGE gels to determine relative *apo-* and *holo-*ACP amounts.  

**ITC experiments**

ITC measurements were performed on a VP-ITC isothermal titration calorimeter (Microcal/GE Healthcare) at 16°C for both Sfp and PptT. CoA analogs and PPTases were diluted to 300 µM and 30 µM, respectively, in 50 mM MES pH 6.0, 250 mM NaCl, 10 mM MgCl₂, and 5% (v/v) glycerol. 30 10 µL injections of 300 µM CoA or rhodamine CoA were added at intervals of 360 seconds while stirring.
at 300 rev. min\(^{-1}\). An initial injection of 2 \(\mu\)L was performed and was not integrated into the data analysis. Data was fit to a titration curve using the built-in Origin software (Microcal).

**BpsA assay of PPTase kinetics**

The BpsA assay was performed in clear 96-well microplates at 25°C. Reaction conditions consisted of 75 mM phosphate pH 7.8, 5 mM MgCl\(_2\), 8 mM ATP, 8 mM L-Gln, 50 nM PPTase, and CoA varying from 500 nM to 250 \(\mu\)M. To this mixture was added BpsA to a final concentration of 1 \(\mu\)M to initiate the reaction. Total reaction volume was 150 uL. Reactions were monitored for change in absorbance at 590 nm for approximately 30 minutes, with intervals of 13-14 seconds per data point. Raw data was analyzed in GraphPad Prism as described previously to obtain kinetic parameters.\(^6\) The BpsA assay for qualitative comparison of mutant and W.T. PptT were carried out under the same conditions as above, except that the PPTase concentration was increased to 5 \(\mu\)M and CoA was held constant at 1 mM in order to visualize activity of weakly active mutants. Reactions were monitored at 590 nm for 45 minutes.

**Fluorescence Polarization**

Fluorescence polarization activity assay proceeded as previously described unless otherwise noted.\(^6\) PptT was implemented at concentrations of 250 nM for inhibitor screening. Substrate concentrations for VibB inhibitor screening was 10 \(\mu\)M carrier protein and 5 \(\mu\)M rhodamine-CoA. Substrate concentrations of 4 \(\mu\)M MAS and 2 \(\mu\)M rhodamine CoA were utilized for inhibitor screening with MAS.

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