Structural and Biochemical Characterization of Compounds Inhibiting Mycobacterium tuberculosis Pantothenate Kinase*

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Background: Pantothenate kinase (PanK), an essential enzyme for Mycobacterium tuberculosis, catalyzes the rate-limiting step in the CoA pathway.

Results: Structures of M. tuberculosis PanK, complexed with new inhibitors, were determined, and their inhibitions were evaluated biochemically.

Conclusion: Inhibitor binding overlaps with the substrate/product sites; also, an alternative mode of ATP binding is proposed.

Significance: These are the first structures of a type I PanK complexed with inhibitors.

Mycobacterium tuberculosis, the bacterial causative agent of tuberculosis, currently affects millions of people. The emergence of drug-resistant strains makes development of new antibiotics targeting the bacterium a global health priority. Pantothenate kinase, a key enzyme in the universal biosynthesis of the essential cofactor CoA, was targeted in this study to find new tuberculosis drugs. The biochemical characterizations of two new classes of compounds that inhibit pantothenate kinase from M. tuberculosis are described, along with crystal structures of their enzyme-inhibitor complexes. These represent the first crystal structures of this enzyme with engineered inhibitors. Both classes of compounds bind in the active site of the enzyme, overlapping with the binding sites of the natural substrate and product, pantothenate and phosphopantothenate, respectively. One class of compounds also interferes with binding of the cofactor ATP. The complexes were crystallized in two crystal forms, one of which is in a new space group for this enzyme and diffracts to the highest resolution reported for any pantothenate kinase structure. These two crystal forms allowed, for the first time, modeling of the cofactor-binding loop in both open and closed conformations. The structures also show a binding mode of ATP different from that previously reported for the M. tuberculosis enzyme but similar to that in the pantothenate kinases of other organisms.

Pantothenate kinase (PanK), EC 2.7.1.33 catalyzes the first and rate-limiting step in the universal CoA biosynthetic pathway, where pantothenate (vitamin B₅) is converted to 4'-phosphopantothenate using ATP as a cofactor (1). Three types of PanK have been described, which differ in their biochemical and structural characteristics. Type I, encoded by the gene coaA (2), is found in a large number of bacterial species and is tightly feedback-regulated by CoA and its thiosteres (3). It is exemplified by the extensively studied Escherichia coli PanK (EcPanK; Ref. 4, 5). The type II enzyme is mostly found in eukaryotes. Humans express four isomers of the enzyme, named PanK1 to 4. Defects in the panK2 gene have been linked to neurodegenerative disease (6, 7). Based on sequence and structural homology some bacterial enzymes, such as Staphylococcus aureus PanK, are also classified as type II enzymes. Whereas the eukaryotic PanKs are feedback-regulated by CoA, the S. aureus enzyme is not (8). Type III, encoded by the gene coaX, is the most widespread type of the enzyme, with homologs present in 12 of the 13 major bacterial groups (9). It is not inhibited by CoA or its thiosteres (10). Many bacteria have two PanK genes coding for different types of the enzyme.

Tuberculosis, caused by the pathogenic bacterium Mycobacterium tuberculosis, is estimated to infect one-third of the world population and is responsible for ~1.4 million deaths annually (World Health Organization, 2011). High-burden countries for tuberculosis are found in sub-Saharan Africa, with high rates of the deadly combination of HIV and tuberculosis. Current treatment of tuberculosis relies on the frontline drugs isoniazid and rifampicin, developed in the 1950s and 1960s. Increasing incidence rates of multidrug-resistant tuberculosis, resistant to front-line drugs, and extensively drug-resistant tuberculosis, resistant to front-line and second-line drugs, make development of new antibiotics targeting M. tuberculosis a global health priority.

The M. tuberculosis genome contains both coaA and coaX genes, coding for a type I and type III PanK, respectively. However, it has been shown that coaX is the only PanK gene essential for bacterial growth in vitro and in vivo (11). The M. tuberculosis type I PanK (MtPanK) is a 312-amino acid (36 kDa) P-loop kinase with a central seven-stranded β-sheet surrounded by α-helices, containing a Walker A motif for nucleo-

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The atomic coordinates and structure factors (codes 4BFS, 4BFT, 4BFU, 4BFV, 4BFW, 4BFX, 4BFY, and 4BFZ) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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2 The abbreviations used are: PanK, pantothenate kinase; MtPanK, M. tuberculosis type I PanK; EcPanK, E. coli PanK; PDB, Protein Data Bank; r.m.s.d., root mean square deviation; CbPanK, Coxella burnetii PanK.
tide binding (12). It functions as a homodimer. MtPanK has been the target for extensive structural studies and has been crystallized in complex with its substrates, products, adenosine or guanosine cofactors, and feedback inhibitor CoA (13–16). The MtPanK structures in this study represent the first complexes with engineered inhibitory compounds and provide a starting point for development of novel antibiotics targeting pathogens that are dependent on type I PanK.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The gene encoding MtPanK (coaA, Rv1092c) was PCR-amplified from genomic DNA, and an N-terminal His	extsubscript{6} tag was added. The resulting construct was ligated into a pET15b expression vector (Novagen) using restriction enzymes Ndel and BamHl. Correct ligations of the sequence coding for a full-length His-tagged MtPanK was confirmed by sequencing. The resulting expression vector was used to transform *E. coli* BL21-AI competent cells (Invitrogen). Cells were incubated at 37 °C in Luria-Bertani growth medium, with 100 μg/ml ampicillin. Expression was induced by adding 0.2% (w/v) L-arabinose, at A	extsubscript{600} = 0.6, and cells were further incubated at 37 °C for 3 h. Cells were centrifuged at 4500 g for 30 min, and the resulting cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole) with 0.01 mg/ml RNase and 0.02 mg/ml DNase. Cells were lysed in a cell disruptor (Constant Systems, Ltd.) and the lysate was centrifuged at 18,000 g for 30 min to remove cell debris. The His-tagged protein was then purified by binding it on a nickel-Sepharose (GE Healthcare) column. The column was washed with 10 column volumes of wash buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 50 mM imidazole), and the protein was eluted with 4 column volumes of elution buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 500 mM imidazole). The elution fractions were pooled and then diluted at a 2:1 ratio with gel filtration buffer (50 mM Hepes, pH 7.5, 300 mM NaCl, 2% (v/v) glycerol, and 10 mM β-mercaptoethanol). The diluted protein sample was further purified using a Superdex 75 (GE Healthcare) size-exclusion column, pre-equilibrated with gel filtration buffer. A modified gel filtration buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 2 mM DTT, and 0.1 mM EDTA) was used in subsequent purifications of the enzyme for biochemical studies. Elution fractions were pooled, and analysis by SDS-PAGE showed it to be essentially the same except that magnesium chloride replaced the calcium. The cryosolution for these crystals consisted of 35% (w/v) PEG 3350, 0.2 M cacodylate, 10 mM MgCl	extsubscript{2} and, where appropriate, 1 mM inhibitor. Crystals were placed in the cryosolution for 5 s before vitrification in liquid nitrogen. Soaking experiments on apo-crystals with this space group were unsuccessful.

**Crystals of a binary complex (MtPanK-1a)** in space group *P*3	extsubscript{1}21 grew in 3–4 days at 20 °C from condition E11 of the JCSG+ screen (Qiagen) containing 14.4% (w/v) PEG 8000, 20% (v/v) glycerol, 0.08 M sodium cacodylate, pH 6.5, and 0.16 M calcium acetate. The optimized crystallization conditions were essentially the same except that magnesium chloride replaced the calcium. The cryosolution for these crystals consisted of 35% (w/v) PEG 3350, 0.2 M cacodylate, 10 mM MgCl	extsubscript{2} and, where appropriate, 1 mM inhibitor. Crystals were placed in the cryosolution for 5 s before vitrification in liquid nitrogen. Soaking experiments on apo-crystals with this space group were unsuccessful.

**Crystals of seven different ternary complexes (MtPanK-inhibitor-Po4)** in space group *P*2	extsubscript{1}2	extsubscript{1}2 were obtained with 1.8 M sodium/potassium phosphate, pH 8.2 (Quik screen, Hampton Research) at 20 °C in a 3–4 days. For optimization of these crystals, the protein concentration was reduced to 5 mg/ml, and the drops were seeded. These drops (0.5 μl each of protein and reservoir solution) were set up manually and equilibrated against 1 ml of the reservoir solution (1.8 M sodium/potassium phosphate, pH 8.2) in Nextal 24-well plates (Qiagen). The ternary complexes were obtained either by cocrystallization or soaking. The crystals (0.1 × 0.2 × 0.2 mm) were vitrified directly from the drops.

**Data Collection and Processing**—Diffraction data from MtPanK, co-crystallized or soaked with inhibitors, were collected from single crystals at the European Synchrotron Radiation Facility (Grenoble, France) or MAXlab (Lund, Sweden). Diffraction data were indexed and integrated using MOSFLM (19) and scaled with SCALA (20). These programs are part of the CCP4 program package (21). Data collection statistics are shown in Table 1.
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Structure Determination and Refinement—The MtPanK-1a binary complex (see Table 1) was crystallized in space group P3₁,21, isomorphous with the published MtPanK-CoA structure (PDB code 2GEV; Ref. 13). These coordinates were stripped of ligands and water molecules and subjected to an initial round of rigid body crystallographic refinement with REFMAC5 (22). The seven structures of the MtPanK-inhibitor-PO₄ ternary complexes (see Table 1) were solved in space group P2₁,2₁,2 were solved by molecular replacement with the program Phaser (23), using the refined MtPanK-1a structure, stripped of ligands and waters, as a search model.

All eight structures were improved by multiple, alternating cycles of crystallographic refinement with REFMAC5 and interactive rebuilding using the program O (24). Waters were added using the carbonyl oxygen profiling methods implemented in O. Coordinates and stereochemical restraints for inhibitory compounds were generated in O, and stereochemical dictionaries for refinement were generated by REFMAC5.

Refinement statistics are shown in Table 1.

Other Methods—Structural figures were created in O and rendered with MOLRAY (25). Detailed structural comparisons were made in O with Ca matching pair cutoffs of 3.8 Å (26). Sequence alignments were made using ClustalW (27), and the corresponding figure was designed with ALINE (28).

RESULTS

Sequence Alignment—The sequence alignment of type I PanK sequences from 13 pathogenic bacterial species shows that the sequences are highly conserved, with a minimum of 41% pairwise sequence conservation (Fig. 1). The longest stretches of conserved residues are found around the P-loop, where the Walker A motif is identical in all 13 species, as are residues involved in substrate and cofactor binding. The Mt enzyme differs from the rest in the loop formed by residues 79–91 (MtPanK numbering), flanked by helix a4 and strand β2, which is extended by four residues. Some variation is seen in the length of the loop formed by residues 203–213 (MtPanK numbering), flanked by strand β6 and helix α10.

The MtPanK-1a Binary Complex—The MtPanK-1a binary complex was solved in space group P3₁,21, with one molecule in the asymmetric unit, and refined to a crystallographic R factor of 19.9% at a resolution of 2.9 Å (see Table 1 for crystallographic data). The crystal structure is in the same space group and has similar unit cell parameters as the previously reported MtPanK structures (discussed in detail below). The open conformation of this loop forces a shift of helix a1 and the loop between helices a1 and a2.

The MtPanK-inhibitor-PO₄ ternary complex dimers superimpose on the various EcPanK or CbPanK dimers with average r.m.s.d. values of 1.3 and 1.2 Å, respectively, with more than ~570 paired Ca atoms. Although the structures of the central β-sheet and the P-loop are highly conserved in all PanK structures, differences can be seen in the surrounding helices and loops. The main differences between the Mt-1, Ec-, and CbPanKs are located to the loop formed by residues 203–213 (MtPanK numbering), flanked by strand β6 and helix a10 (Fig. 1). This loop is elongated by seven residues in the Ec and Cb enzymes and extends away from the center of the molecule in different conformations (Fig. 2). However, the loop formed by residues 79–91 (MtPanK numbering), flanked by helix a4 and strand β2, is elongated by four residues in the Mt enzyme (Figs. 1 and 2).

Binding of Inhibitors—We have determined crystal structures of eight protein-inhibitor complexes of MtPanK with inhibitors of two different classes. Compounds 1a to 1f are triazoles, whereas compounds 2a and 2b are biaryls (Table 2). Electron density is well defined for all of these inhibitors, except for the slightly less well defined electron density of the difluorophenyl ring in 1f (Fig. 3).

The binding mode of the triazole compounds is exemplified by triazole 1a in Fig. 4a. They overlap with pantothenate (MtPanK-Pan-AMPPCP, PDB code 2ZSE; Ref. 14), and the

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1. C. Franklin, J. Cheung, M. Rudolph, M. Cassidy, E. Gary, F. Burshteyn, and J. Love, unpublished work.
FIGURE 1. Sequence alignment of type I PanKs from various pathogenic bacteria. MtPanK numbering and secondary structure are shown as defined by Das et al. (13). The blue triangle shows residue Arg-238, interacting with the phosphate bound in the P-loop, or inhibitory compounds. Red triangles show residues involved in pantothenate/phosphopantothenate binding. Green triangles show residues involved in adenine binding in the nucleoside binding site reported for Ec- and CbPanK. Sequences used in this alignment were as follows: MtPanK (UniProt ID P63810), Corynebacterium diphtheriae PanK (UniProt ID Q6NI48), Escherichia coli PanK (UniProt ID P0A6I3), Salmonella typhi PanK (UniProt ID Q8Z318), Yersinia pestis PanK (UniProt ID Q32aF0), Haemophilus influenzae PanK (UniProt ID Q9KV38), Vibrio cholerae PanK (UniProt ID Q8Y8I0), Listeria monocytogenes PanK (UniProt ID P44793), and Streptococcus pneumoniae PanK (UniProt ID Q97RH6).
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Table 1

Data collection and refinement statistics

| MtPanK-1a | MtPanK-1c-PO₄ | MtPanK-1d-PO₄ | MtPanK-1e-PO₄ | MtPanK-1f-PO₄ | MtPanK-2a-PO₄ | MtPanK-2b-PO₄ |
|-----------|---------------|---------------|---------------|---------------|---------------|---------------|
| PDB code  | 4BFS          | 4BFU          | 4BFV          | 4BFW          | 4BFY          | 4BFZ          |
| Beam line | 1911-2 MAXlab | 1914-1 ESRF   | 1914-1 ESRF   | 1914-1 ESRF   | 1914-1 ESRF   | 1914-1 ESRF   |
| Detector  | MAR 165       | MAR 165       | MAR 165       | MAR 165       | MAR 165       | MAR 165       |
| Wavelength (Å) | 1.047          | 1.047          | 1.047          | 1.047          | 1.047          | 1.047          |
| Space group | P2₁2₁2₁      | P2₁2₁2₁      | P2₁2₁2₁      | P2₁2₁2₁      | P2₁2₁2₁      | P2₁2₁2₁      |
| Cell axial lengths (Å) | 105.28, 105.28, 90.80 | 88.40, 149.69, 62.77 | 88.60, 150.69, 63.18 | 88.33, 151.77, 63.13 | 89.03, 150.71, 60.50 | 88.01, 149.51, 62.83 |
| Resolution range (Å) | 30.0–290 (3.06–2.90) | 40.0–2.29 (2.41–2.28) | 60.0–2.28 (2.40–2.28) | 40.0–2.29 (2.42–2.29) | 50.0–2.27 (2.39–2.27) | 60.0–2.70 (2.85–2.70) |
| No. of reflections measured | 124,322 (17,252) | 136,311 (19,054) | 266,498 (38,455) | 280,048 (40,356) | 125,447 (18,679) | 148,478 (21,019) |
| No. of unique reflections | 13,018 (1,865) | 37,958 (5,787) | 39,320 (5,655) | 38,825 (5,589) | 38,286 (5,516) | 23,491 (3,392) |
| Average multiplicity | 6.6 (6.0) | 36 (3.3) | 39 (3.3) | 38 (3.3) | 39 (3.3) | 39 (3.3) |
| Completeness (%) | 98.9 (95.5) | 99.4 (92.9) | 99.3 (93.3) | 99.3 (93.3) | 100 (100) | 99.6 (99.7) |
| Rmerge | 0.115 (0.491) | 0.111 (0.486) | 0.085 (0.430) | 0.095 (0.399) | 0.102 (0.500) | 0.114 (0.495) |
| Rmerge,ref | 0.127 (0.537) | 0.130 (0.571) | 0.093 (0.466) | 0.103 (0.430) | 0.122 (0.500) | 0.148 (0.541) |
| Rmerge,free | 0.113 (0.331) | 0.066 (0.291) | 0.036 (0.176) | 0.039 (0.159) | 0.066 (0.316) | 0.099 (0.215) |
| Rmerge,free (%) | 10.1 (3.3) | 10.1 (2.6) | 12.3 (3.7) | 19.2 (5.0) | 10.6 (3.4) | 9.3 (3.2) |

Refinement statistics

| MtPanK-1a | MtPanK-1c-PO₄ | MtPanK-1d-PO₄ | MtPanK-1e-PO₄ | MtPanK-1f-PO₄ | MtPanK-2a-PO₄ | MtPanK-2b-PO₄ |
|-----------|---------------|---------------|---------------|---------------|---------------|---------------|
| Resolution range (Å) | 30.0–290 (2.98–2.90) | 40.0–2.29 (2.35–2.29) | 60.0–2.28 (2.33–2.28) | 40.0–2.29 (2.35–2.29) | 50.0–2.27 (2.33–2.27) | 60.0–2.70 (2.85–2.70) |
| No. of reflections used in working set | 12,375 (807) | 36,047 (2,420) | 37,299 (2,497) | 36,854 (2,466) | 36,326 (2,446) | 22,338 (1,453) |
| Best R value for Rfree calculation | 19.9 | 22.5 | 22.1 | 20.6 | 21.6 | 28.1 |
| Rfree (%) | 24.5 | 28.6 | 26.3 | 26.2 | 27.3 | 25.7 |
| No. of non-hydrogen atoms | 2,848 | 5,109 | 5,148 | 5,229 | 5,164 | 5,075 |
| No. of solvent waters | 13 | 99 | 100 | 99 | 100 | 100 |
| Average B-factors (Å²) | 52.0 | 45.4, 43.1 | 74.3 | 48.8 | 32.0, 32.1 | 42.7, 46.3 |
| Protein atoms (A, B) | 52.0 | 45.4, 43.1 | 74.3 | 48.8 | 32.0, 32.1 | 42.7, 46.3 |
| R.m.s.d. from ideal bond length (Å) | 0.009 | 0.012 | 0.011 | 0.010 | 0.012 | 0.009 |
| Phosphate ion (A, B) | 0.009 | 0.012 | 0.011 | 0.010 | 0.012 | 0.009 |
| Phosphate ion (A, B) | 0.009 | 0.012 | 0.011 | 0.010 | 0.012 | 0.009 |
| r.m.s.d. from ideal bond angle (°) | 1.479 | 1.975 | 1.650 | 1.612 | 1.897 | 1.654 |

<sup>a</sup> ESRF indicates the European Synchrotron Radiation Facility.

<sup>b</sup> Values were calculated using methods described by Matthews (Ref. 31).

<sup>c</sup> Values in parentheses refer to the outer resolution shell.

<sup>d</sup> Values were calculated using crystallographic R factors generated by the computer programs described in the Experimental Procedures.
pantothenate moieties of phosphopantothenate (MtpPanK-PPan-ADP, PDB code 2ZSA; Ref. 14) and CoA (MtpPanK-CoA, PDB code 2ZSD) in the active site pocket (Fig. 5, a and b, respectively). These compounds fan out from the conserved triazole ring to form similar U-shaped conformations where the halogen-substituted phenyl rings are roughly co-planar, with ring edges abutting. In the 1b complex, for example, the vector normals to the phenyl rings form a 51° angle, with ring centers separated by 7.8 Å, and the pair of ring edge carbons separated by ~5.5–6.5 Å. Each triazole ring forms a pair of conserved hydrogen bond interactions with side chains that are themselves highly conserved (Fig. 4a). Residues Tyr-235 and Asn-277 are involved in pantothenate and phosphopantothenate binding (14), respectively, but here, the hydroxyl group of Tyr-235 forms a hydrogen bond to one nitrogen in the triazole ring, whereas the amide group of the side chain of Asn-277 hydrogen bonds to another (Fig. 4a). Hydrogen bonding atoms in the peptide linkage make interactions with water molecules, where visible, except in the 1c complex where the carbonyl oxygen directly interacts with the hydroxyl of Tyr-177. No hydrogen bonding interactions are observed to the ether-linking oxygen. The removal of the ether-linked oxygen in 1c is easily accommodated by the inhibitor. Here, we see a ~0.7 Å shift in the fluorophenyl group. This involves a small pivot of the triazole

![Diagram](image_url)

**FIGURE 2.** Cα superposition of A molecules of all MtpPanK-inhibitor-PO₄ complexes (red), MtpPanK-1a (green), EcPanK-CoA (light blue, PDB code 1ESM), EcPanK-Pan-ADP (blue, PDB code 1SQ5), and CbPanK-ADP (yellow, PDB code 3TQC). Shown is the conserved conformation of the P-loop, the open and closed form of the adenosine lid (a-lid), the alternative conformation of helix α4, and the two loops (with MtpPanK numbering) elongated in MtpPanK, EcPanK, or CbPanK.

**TABLE 2**

Structures of the inhibitory compounds and results from the biochemical assay, thermal shift, and MIC measurements

| Structure | Class | Compound ID | IC₅₀ at Kᵣ for ATP (µM) | IC₅₀ at 50x Kᵣ for ATP (µM) | Mt MIC (µg/ml) | Tm shift with 0.0 mM ATP (°C) | Tm shift with 0.5 mM ATP (°C) | Tm shift with 1.0 mM ATP (°C) | Mode of Inhibition |
|-----------|-------|-------------|-------------------------|---------------------------|----------------|-----------------------------|-------------------------------|----------------------|------------------|
| ![Triazole 1a](image_url) | Triazole | 1a          | 0.09                    | 5.5                       | >64            | 3.8                         | 1.1                           | 0.9                  | Competitive      |
| ![Triazole 1b](image_url) | Triazole | 1b          | 1.13                    | >12.5                     | >64            | 1.8                         | 0.4                           | 0.3                  | Competitive      |
| ![Triazole 1c](image_url) | Triazole | 1c          | 0.15                    | 10.36                     | >64            | 3.7                         | 1.8                           | 0.4                  | Competitive      |
| ![Triazole 1d](image_url) | Triazole | 1d          | 0.14                    | 2.82                      | >32            | 3.9                         | 1.7                           | 1.8                  | Competitive      |
| ![Triazole 1e](image_url) | Triazole | 1e          | 0.05                    | 0.69                      | >32            | 4.6                         | 2.5                           | 2.9                  | Competitive      |
| ![Triazole 1f](image_url) | Triazole | 1f          | 1.09                    | 14.0                      | >64            | 2.4                         | 1.0                           | 0.7                  | Competitive      |
| ![Biaryl 2a](image_url) | Biaryl | 2a          | 0.12                    | 0.13                      | >64            | 2.4                         | 4.8                           | 4.6                  | Non-competitive  |
| ![Biaryl 2b](image_url) | Biaryl | 2b          | 0.55                    | ND                        | >64            | ND                          | ND                            | ND                   | Non-competitive  |
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The biaryl compounds, 2a and 2b (Fig. 4b), were both crystallized in ternary complexes with phosphate ions in the orthorhombic space group. These compounds also bind in the active site pocket, overlapping with the binding site of the triazole compounds (overlaid structure, Fig. 4c), and that of the pantothenate moieties in the previous MtPanK structures (Fig. 5, a and b). In the 2b complex, the Arg-238 side chain interacts with the phosphate ion as seen with the triazoles but changes conformation to form a salt link with the carboxylate of the inhibitor in the 2a complex. The Tyr-182 side chain has the same rotamer conformation observed in the 1c complex such that the hydroxyl group interacts with the ether linkage oxygen atom, as well as the carboxylate of one inhibitor and the amide nitrogen of the other (Fig. 4b). The para-nitrophenyl group from each inhibitor packs into the hydrophobic tunnel used by the para-fluorobenzyl ring of 1e. The conserved residues Tyr-235 and Asn-277 that are important for triazole binding do not interact directly with the biaryl class of compounds. Instead, they form hydrogen bonds to a water molecule that, in turn, interacts with a nitrogen of the piperazine ring. The 2-pyridyl nitrogen also forms a hydrogen bond to another water molecule, structurally equivalent to the water forming the same interaction with the peptide oxygen of the triazoles (Fig. 4b). When the triazole and biaryl complexes are superimposed on the ATP analogue complexes of Ec and MtPanK, the phosphate ion that we observe in the orthorhombic crystal form overlaps with the ATP β-phosphate group (Fig. 5c).

The Different Conformations of the Adenosine Lid—A structural feature of type I PanK enzymes is an adenosine-lid loop formed by residues 38–44 (MtPanK numbering) and flanked by helices α2 and α3 (Figs. 1 and 2). The loop is in an open form when the adenosine moiety of ADP or ATP is bound, exemplified by the EcPanK-Pan-ADP, EcPanK-AMP, and CbPanK-ADP complex structures (Fig. 6a). In the absence of a bound adenosine, the loop adopts a closed conformation, exemplified by the EcPanK-CoA structure (Fig. 6b). When switching from an open to closed conformation the loop shifts ~5 Å toward the adjacent helix α11. To accommodate this shift, the flanking helix α2 unwinds, contracting by one turn in the closed conformation. Helix α2 extends almost to helix α3 in the open form of the CbPanK-ADP structure. The adenine ring of the cofactor is stacked between the loop and conserved residue His-302 (MtPanK numbering), which in turn stacks with the ring of conserved residue Trp-234 (MtPanK numbering) in the open form of the EcPanK and CbPanK structures (Fig. 6a). However, the MtPanK structures crystallized in complex with nucleoside cofactors exhibit an alternative binding-mode (Fig. 5c; Refs. 15 and 16), where the adenine or guanine moieties occupy roughly the same binding site as the adenine moiety of CoA (Fig. 5b). In these structures, the adenosine lid retains a closed conformation, unable to accommodate a nucleoside in this binding site. In the MtPanK-1a complex, crystallized in the previously reported trigonal space group, the adenosine lid

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**FIGURE 3.** Representative electron density for inhibitors in the A molecules of their respective complex structure. The αA-weighted (2Fo–DFo) electron density maps (34) are drawn at the level of the root mean square value of the respective map.
adopts a closed conformation, identical to all previously reported MtPanK structures. However, in the MtPanK-inhibitor-PO₄ structures crystallized in the orthorhombic space group, the loop adopts an open conformation. This conformation is similar to the open conformation of the EcPanK and CbPanK structures, although no adenosine is present in the adenosine-binding site (Fig. 6).

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FIGURE 4. a, binding of a triazole compound (gray carbons) in the active site, exemplified by the MtPanK-1a complex. Also shown are the residues involved in compound interactions and the P-loop. b, binding of a biaryl compound (white carbons) in the A molecule active site, exemplified by the MtPanK-2b-PO₄ complex. Also shown are the residues involved in compound interactions (yellow carbons) and the phosphate ion bound in the P-loop. c, superposition of all triazole compounds (gray carbons) and biaryl compounds (white carbons) in the MtPanK active site. The second-ary structure is colored as in Fig. 1.

FIGURE 5. a, superposition of MtPanK-1a, MtPanK-2b-PO₄, MtPanK-Pan-AMP-PCP (PDB code 2ZSE) and MtPanK-Pan-ADP (PDB code 2ZSA) showing the triazole compound 1a (green), the biaryl-compound 2b (red) with the phosphate ion (atom colors), pantothenate (blue), and phosphopantothenate (yellow). b, superposition of MtPanK-1a, MtPanK-2b-PO₄, and MtPanK-CoA (PDB code 2ZSD) showing the triazole compound 1a (green), the biaryl compound 2b (red) with the phosphate ion (atom colors), and CoA (light blue). c, superposition of MtPanK-1a, MtPanK-2b-PO₄, MtPanK-Pan-AMP-PCP (PDB code 2ZSE) and EcPanK-AMPPNP (PDB code 1ESN) showing the triazole compound 1a (green), the biaryl compound 2b (red) with the phosphate ion (atom colors), AMPPCP bound in the Mt structure (gray), and AMPPNP bound in the Ec structure (magenta). The van der Waals radii of the fluorine in compound 1a and the γ-phosphate oxygen of AMPPCP and AMPPNP are shown as transparent spheres.
Attempts to obtain binary complexes of MtPanK with ATP or ternary complexes with inhibitory compounds and ATP, whether by co-crystallization or soaking in conditions for the orthorhombic space group, were unsuccessful. Non-hydrolyzable ATP analogues, adenosine, adenosine ribose, or ADP were also used in place of ATP but without success. Although some crystals were obtained and diffraction data were collected, the resolution and data quality did not allow modeling of the entities seen in the active site. The high phosphate concentration required for crystallization in this space group, resulting in a phosphate occupying the P-loop or potentially close contacts required for crystallization in this space group, resulting in a structural rearrangement such that the ring to ring centers are closer in range 3.3–3.7 Å. The trifluoromethyl group points toward the imidazole ring of His-179, with closest contacts in the range 3.3–3.7 Å. The trifluoromethyl group points toward the edge of the second phenyl ring of each inhibitor to form potentially stabilizing multipolar interactions. The combined effects may be sufficient to explain the 10-fold increase in IC_{50} values that are observed for other, potentially stabilizing multipolar interactions. The structural data have been used to clarify the nature of fluorine-protein interactions. It has been suggested that C-F dipolar and multipolar interactions with protein side chain and main chain atoms can benefit the energetics of inhibitor binding (30). Favorable interactions with backbone and side chain amides as well as the guanidinium group of arginine residues appear to be common occurrences. The top four, most active triazoles (1a, 1c, 1d, and 1e) contain a trifluoromethyl substitution that is positioned close to the imidazole ring of His-179, with closest contacts in the range 3.3–3.7 Å. The trifluoromethyl group points toward the edge of the second phenyl ring of each inhibitor to form other, potentially stabilizing multipolar interactions. The combined effects may be sufficient to explain the 10-fold increase in IC_{50} values that are observed for 1b and 1f where the trifluoromethyl group has been substituted. In seven of the eight triazoles, the second phenyl ring is also fluorinated at the para-position. In these structures, we observe an interaction with the guanidinium group of Arg-238, but the replacement of the para-fluorophenyl ring by an ortho-methylphenyl ring in 1d results in a structural rearrangement such that the ring to ring centers are closer in 1d.

**IC_{50}, Minimum Inhibitory Concentration, and Thermal Stability Measurements**—Detailed results for related inhibitory compounds of the classes presented here have been described (17). The loss of inhibition of the triazole compounds when the assay was run in excess of ATP (Table 2) indicates that these compounds compete with ATP. This is supported by the thermal stability measurements of these compounds where a decrease in the thermal shift is observed when the measurements are performed at higher ATP concentrations (Table 2).

The biaryl compound 2a shows comparable levels of inhibition at low and high ATP concentrations, suggesting that this class of compounds is non-competitive with respect to ATP. The thermal stability measurements for this compound show an increase in thermal shift in the presence of ATP, also indicative of a non-competitive mode of inhibition. Unfortunately, inhibition in the presence of ATP and thermal stability measurements were not available for compound 2b. None of the compounds described here inhibited growth of Mt in the whole cell assay at their respective maximum concentration tested (Table 2).

**DISCUSSION**

We have determined the first enzyme-inhibitor complex structures of a type I PanK enzyme. The structures show that the two classes of inhibitory compounds, triazoles and biar-
with helix α7 of a symmetry-related molecule (Fig. 7). In particular, the guanidinium group of residue Arg-38 in the adenosine lid forms a hydrogen bond to the carbonyl oxygen of residue Arg-140 in helix α7 of the symmetry-related molecule. These crystal packing interactions are present in all MtPanK structures solved in crystal form II but not in the two structures solved in crystal form I (MtPanK-CoA, PDB codes 2GES and 2GET). Crystal packing interactions with the adenosine lid are also absent in our structures solved in the orthorhombic space group, where the adenosine lid adopts an open conformation. In contrast, the complex structures with nucleoside cofactors were crystallized in crystal form II of the trigonal space group, where the adenosine lid assumes a closed conformation. In these structures, the adenosine or guanosine moieties occupy roughly the same binding site as the adenosine moiety of CoA instead of binding in the adenosine binding site (Fig. 5, b and c).

Crystal contacts with the adenosine lid in these structures may be responsible for keeping the adenosine lid in a closed conformation, forcing the nucleoside cofactors to adopt an alternative binding mode. To date, no crystal structure of a nucleoside-bound enzyme has been solved in the orthorhombic space group or crystal form I of the trigonal space group. However, the conserved nature of the adenosine binding site and the ability of MtPanK to adopt both open and closed forms of the adenosine-lid suggest that the same mode of nucleoside binding as seen in the Ec and Cb enzymes is biologically relevant.

Even though the compounds described in this study were inactive against Mt in the whole cell assay, they are potent inhibitors of MtPanK. The structural information from this study provides us with tools for structure-based optimization of these compounds and to design novel inhibitors that are active against this target in other pathogens.

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