The crystal structure of the drug target *Mycobacterium tuberculosis* methionyl-tRNA synthetase in complex with a catalytic intermediate

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Mycobacterium tuberculosis is a pathogenic bacterial infectious agent that is responsible for approximately 1.5 million human deaths annually. Current treatment requires the long-term administration of multiple medicines with substantial side effects. Lack of compliance, together with other factors, has resulted in a worrisome increase in resistance. New treatment options are therefore urgently needed. Here, the crystal structure of methionyl-tRNA synthetase (MetRS), an enzyme critical for protein biosynthesis and therefore a drug target, in complex with its catalytic intermediate methionyl adenylate is reported. Phenylalanine 292 of the *M. tuberculosis* enzyme is in an ‘out’ conformation and barely contacts the adenine ring, in contrast to other MetRS structures where ring stacking occurs between the adenine and a protein side-chain ring in the ‘in’ conformation. A comparison with human cytosolic MetRS reveals substantial differences in the active site as well as regarding the position of the connective peptide subdomain 1 (CP1) near the active site, which bodes well for arriving at selective inhibitors. Comparison with the human mitochondrial enzyme at the amino-acid sequence level suggests that arriving at inhibitors with higher affinity for the mycobacterial enzyme than for the mitochondrial enzyme might be achievable.

1. Introduction

Caused by the bacillus *Mycobacterium tuberculosis*, tuberculosis (TB) is an infectious disease that typically affects the lungs (pulmonary TB) and is fatal if untreated (Centers for Disease Control and Prevention, 2011). TB is one of the top ten causes of death worldwide (World Health Organization, 2017). In 2016, there were an estimated 10.4 million new TB cases. Of those cases, approximately one million were children. People infected with HIV, as well as other immunocompromised individuals, are especially susceptible to TB, and accounted for 0.4 million deaths in 2016. Globally, 1.7 million people died from tuberculosis in 2016 (World Health Organization, 2017). The recommended treatment for drug-susceptible TB is a six-month regimen starting with two months of four first-line drugs: ethambutol, isoniazid, pyrazinamide and rifampicin (World Health Organization, 2017). This treatment is associated with significant side effects such as hepatotoxicity, CNS toxicity, exanthema, arthralgia and nausea, which are important factors in lack of compliance (Schaberg *et al.*, 1996). Although TB treatment has saved millions of lives, the crisis of multidrug-resistant TB (MDR-TB) treatment is a major global health concern. In 2016 there

Table 1

Structures of methionyl-tRNA synthetases referred to in this study.

PDB code	Organism	Ligand(s)	Crystallized MetRS	Reference
6ax8	<i>Mycobacterium tuberculosis</i>	Met-AMP	Full length	This publication
2x1m	<i>Mycobacterium smegmatis</i>	L-Methionine	Full length	Ingvarsson & Unge (2010)
2x1l	<i>Mycobacterium smegmatis</i>	L-Methionine and adenosine	Full length	Ingvarsson & Unge (2010)
2ct8	<i>Aquifex aeolicus</i>	Met-AMP and tRNA ^{Met}	Full length	Nakanishi <i>et al.</i> (2005)
4eg3	<i>Trypanosoma brucei</i>	Met-AMP	237–773	Koh <i>et al.</i> (2012)
5gl7	<i>Homo sapiens</i> , cytosolic	None	221–834	H. Y. Cho, H. J. Lee & B. S. Kang (unpublished work)
3kfl	<i>Leishmania major</i>	Met-AMP and PP _i	206–747	Larson <i>et al.</i> (2011)

were an estimated 600 000 patients with rifampicin-resistant TB (RR-TB), of which 490 000 had MDR-TB (World Health Organization, 2017). On top of this, there are drug–drug interactions between anti-HIV drugs and other chronic disease medications for some of the current TB drugs (Koul *et al.*, 2011). Hence, there is an urgent need for new drug candidates to fight the scourge of TB.

As demonstrated by the action of aminoglycosides, tetracyclines, macrolides, streptogramins and phenicols, interfering with bacterial protein synthesis has been shown to be extremely effective for the development of antibiotics (Kohanski *et al.*, 2010; Wilson, 2014). Aminoacyl-tRNA synthetases (aaRSs) are a major group of proteins that are essential for protein synthesis. AaRSs catalyze a two-step esterification reaction in which each amino acid (aa) is paired with its cognate tRNA, forming an aminoacyl-tRNA (Ibba & Söll, 2000; Sheppard *et al.*, 2008):

In the first, ATP-dependent step a highly reactive aminoacyl adenylate (aa~AMP) intermediate is formed and an inorganic pyrophosphate is released. In the second step, the amino-acid moiety is transferred from the activated intermediate to the 3'-end of the tRNA, yielding an aminoacyl-tRNA as the product and AMP as the leaving group (Ibba & Söll, 2000).

AaRSs have been validated as antimicrobial drug targets (Ochsner *et al.*, 2007; Schimmel *et al.*, 1998). Anti-aaRS compounds can block the ATP- and/or the amino-acid-binding site, the tRNA-recognition site, an allosteric site or an editing site (Fang, Yu *et al.*, 2015). The widely used antibiotic mupirocin is in clinical topical use against *Staphylococcus aureus* infections and binds both the ATP- and isoleucine-binding sites of *S. aureus* isoleucyl-tRNA synthetase (IleRS; Nakama *et al.*, 2001). However, *M. tuberculosis* IleRS is not inhibited by mupirocin and therefore *M. tuberculosis* is naturally resistant to this antibiotic (Sassanfar *et al.*, 1996).

Methionyl-tRNA synthetase (MetRS) is particularly interesting since it recognizes both the initiator tRNA_i^{Met} and the elongator tRNA^{Met} delivering methionine during translation. Depending on their structural features and conserved motifs, aaRSs can be separated into two classes (Deniziak & Barciszewski, 2001). MetRS belongs to the class I aaRSs, which are characterized by the amino-acid sequence motifs HIGH and KMSKS and a classical Rossmann fold as part of the catalytic domain. Together with arginyl-, cysteinyl-, isoleucyl-, leucyl-

and valyl-tRNA synthetases, MetRS belongs to subclass Ia, with the HIGH and KMSKS motifs corresponding to ¹⁸HVGH²¹ and ²⁹⁹KMSKS³⁰³ in *M. tuberculosis* MetRS (*Mtub*MetRS). MetRSs can be categorized into two major forms, MetRS1 and MetRS2, depending on structural features and susceptibility to inhibitors. Structurally, MetRS1 orthologs contain one 'knuckle' motif with either one or no Zn²⁺ ion bound. MetRS2 enzymes contain two knuckle motifs with either one or two Zn²⁺ ions bound. Most Gram-positive bacterial genera harbor the MetRS1 form, while most Gram-negative genera contain the MetRS2 form (Gentry *et al.*, 2003; Green *et al.*, 2009). A few bacteria harbor both forms (Brown *et al.*, 2003; Gentry *et al.*, 2003). Eukaryotic organisms contain both forms, with the mitochondrial enzyme exhibiting MetRS1 features and the cytosolic enzyme being of the MetRS2 form (Green *et al.*, 2009).

Highly effective compounds that inhibit MetRSs from pathogenic trypanosomatid protozoa have been reported by our group (Pedro-Rosa *et al.*, 2015; Shibata *et al.*, 2011, 2012). These reports focused mainly on MetRS from *Trypanosoma brucei*, the causative agent of human African trypanosomiasis (HAT), also known as sleeping sickness. Crystal structures of *T. brucei* MetRS synthetase (*Tbru*MetRS) in complex with various inhibitors (Huang *et al.*, 2016, 2017; Koh *et al.*, 2012, 2014; Zhang *et al.*, 2016) have guided the design of new anti-trypanosomatid compounds with a low nanomolar inhibitory potency on the enzyme and parasites, and a steadily improved pharmacokinetic profile. This series of inhibitors has also been optimized and tested for their effect on MetRS from *Staphylococcus aureus* (*Saur*MetRS) and other Gram-positive bacteria, with minimum inhibitory concentrations (MICs) for some compounds of less than 0.02 µg ml⁻¹ (Faghih *et al.*, 2017).

A major structural biology effort has been made by many investigators to create a platform of crystal structures of key *M. tuberculosis* proteins to assist in the development of new therapeutic agents to treat tuberculosis patients (*e.g.* Murillo *et al.*, 2007; Fang, van der Merwe *et al.*, 2015; Terwilliger *et al.*, 2003). Here, we describe the crystal structure of *M. tuberculosis* MetRS (*Mtub*MetRS) at 2.6 Å resolution in complex with the intermediate methionyl adenylate (Met-AMP). Various structural features are compared with those of other MetRSs with known structure. Striking conformational changes near the active site are evident for both the KMSKS loop and the connective peptide subdomain 1 (CP1; Table 1 provides information on the MetRS structures used for comparisons in this paper). In the Met-AMP binding region of the human cytosolic and mitochondrial enzymes several amino-acid

Table 2

Crystallographic data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

PDB code	6ax8
Data collection	
Space group	<i>H3</i>
<i>a</i> , <i>b</i> , <i>c</i> (Å)	196.96, 196.96, 39.18
Resolution (Å)	38.18–2.60 (2.72–2.60)
<i>R</i> _{merge}	0.198 (1.085)
<i>R</i> _{p.i.m.}	0.081 (0.526)
Observed reflections	91700 (11269)
Unique reflections	17391 (2110)
Mean <i>I</i> / σ (<i>I</i>)	7.3 (2.3)
Multiplicity	5.3 (5.3)
Wilson <i>B</i> factor (Å ²)	33.9
Completeness (%)	99.9 (99.9)
<i>CC</i> _{1/2}	0.989 (0.709)
Refinement	
Resolution (Å)	38.18–2.60
Reflections used	16441
<i>R</i> _{work} / <i>R</i> _{free}	0.237/0.246
No. of atoms	
Protein	3980
Met-AMP	31
Water	38
No. of residues	509
Average <i>B</i> factors (Å ²)	
All atoms	60.7
Protein	60.5
Met-AMP	95.6
Water	51.8
R.m.s. deviations	
Bond lengths (Å)	0.009
Bond angles (°)	1.29
Ramachandran plot†	
Favored (%)	97
Outlier (%)	0
Met-AMP	
LLDF‡	1.13
RSR§	0.24

† Ramachandran plot statistics as reported by the wwPDB validation report. ‡ Local ligand density fit as reported by the wwPDB validation report. § Real-space *R* value as reported by the wwPDB validation report.

differences occur compared with *M. tuberculosis* MetRS, indicating that there are opportunities to arrive at inhibitors which have a higher affinity for the *M. tuberculosis* enzyme than for the human homologs.

2. Materials and methods

2.1. Expression and purification of *MtubMetRS*

MtubMetRS was cloned into the AVA0421 vector (Alexandrov *et al.*, 2004; Choi *et al.*, 2011; Mehlin *et al.*, 2006) and expressed in *Escherichia coli* for subsequent purification. The protein was purified using a Ni-NTA affinity column (Qiagen, Valencia, California, USA) followed by size-exclusion chromatography (SEC) on a Superdex 75 column (Amersham Pharmacia Biotech) using SEC buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 2 mM DTT). The purity of *MtubMetRS* was assessed by SDS-PAGE and the protein concentration was determined using the Bio-Rad protein-assay dye reagent (Bio-Rad) based on the Bradford method (Bradford, 1976). A final yield of 1.3 mg pure *MtubMetRS* per litre of *E. coli* culture was obtained and the

protein was concentrated to about 8 mg ml^{−1} for crystallization.

2.2. Crystallization of *MtubMetRS*

Purified *MtubMetRS* was prepared for crystallization by the addition of 10 mM MgATP, 10 mM methionine and 1 mM TCEP (protein solution). Crystals were obtained after one month at room temperature by vapor diffusion using sitting drops equilibrated against a reservoir consisting of 30–34% PEG 8000, 150–200 mM ammonium sulfate, 100 mM sodium cacodylate pH 6.5. The drops consisted of 1 µl *MtubMetRS* protein solution and 1 µl reservoir solution. After growth, crystals were picked up in a loop (Teng, 1990), flash-cooled in liquid nitrogen (Haas & Rossmann, 1970) in cryosolution (30% glycerol in reservoir solution) and stored until data collection.

2.3. Data collection and structure determination

Data were collected under cryogenic conditions on beamline 12-2 at the Stanford Synchrotron Radiation Lightsource (SSRL) at a wavelength of 0.98 Å. Many crystals exhibited a considerable degree of twinning. Only a single crystal was not twinned and diffracted to a higher resolution than all others. This crystal allowed the eventual structure determination. *HKL-2000* (Otwinowski & Minor, 1997) was used for data processing. The structure of *M. smegmatis* MetRS (Ingvarsson & Unge, 2010; PDB entry 2x1l) was used as a model to obtain initial phases by molecular replacement using *Phaser* (McCoy *et al.*, 2007). Iterations of manual building and rebuilding using *Coot* (Emsley *et al.*, 2010) were alternated with refinement of the structure with *REFMAC5* (Murshudov *et al.*, 2011). *MolProbity* (Chen *et al.*, 2010) was used throughout the process for structure validation. Data-collection and crystallographic refinement statistics are given in Table 2. The figures were created with *PyMOL* (v.1.7; Schrödinger; <https://www.pymol.org>).

3. Results and discussion

3.1. The structure of the *M. tuberculosis* MetRS-Met-AMP binary complex

We solved the structure of *M. tuberculosis* MetRS (*MtubMetRS*), a 58 kDa monomeric enzyme, in complex with its intermediate product methionyl adenylate (Met-AMP). Despite major efforts, no co-crystals of *MtubMetRS* in complex with our available MetRS inhibitors could be obtained. The purified enzyme was incubated with the substrates methionine and ATP prior to setting up crystallization trays. The electron-density map revealed the presence of Met-AMP in the active site of the enzyme. The *MtubMetRS*-Met-AMP complex crystal structure, where the symbol ‘·’ indicates a noncovalent complex, was determined at 2.6 Å resolution (Table 2). There is one molecule in the asymmetric unit and, except for the final ten residues at the C-terminus, every residue could be built into well defined electron density.

Several structural elements can be distinguished in *Mtub*-MetRS (Fig. 1): (i) a Rossmann-fold catalytic domain (CD) with (ii) an inserted connective peptide domain (CP), (iii) a stem-contact fold domain (SCF) containing the conserved KMSKS motif and (iv) an anticodon-binding domain (ABD). The SCF and ABD are connected by a linking π 1-helix between helices α 14 and α 15.

The *Mtub*MetRS CD is predominantly an α/β domain that extends from the N-terminal end of the protein to residue His290 and is interrupted by the CP, which comprises residues Ile116–Tyr226. The CP comprises two subdomains: subdomain CP1 formed by the antiparallel β -strands β 4 and β 8, and subdomain CP2 formed by helices α 5 and α 6. Subdomain CP1 in *Mtub*MetRS is in the closed conformation, as seen previously in the structure of *Msm*MetRS and other MetRS structures (Ingvarsson & Unger, 2010). CP1 harbors one ‘knuckle’ devoid of residues able to coordinate Zn^{2+} , which identifies *Mtub*MetRS as a member of the MetRS1 form (Gentry *et al.*, 2003; Green *et al.*, 2009).

The SCF connects the CD and ABD. The SCF harbors the KMSKS loop which contains, using *M. tuberculosis* sequence numbering, the conserved motif $^{299}\text{KMSKS}^{303}$. Interestingly, when superimposing the *Mtub*MetRS structure with the structure of the homologous *M. smegmatis* MetRS (*Msm*MetRS), which shares 76% sequence identity overall and 94% identity in the Met-AMP-binding site, the KMSKS loop adopts dramatically different conformations in these two closely related tRNA synthetases. The KMSKS loop in *Msm*MetRS, in the presence of either methionine (PDB entry 2x1m; Ingvarsson & Unger, 2010) or of methionine and adenosine (PDB entry 2x1l; Ingvarsson & Unger, 2010), is in a wide-open conformation (Fig. 2). In contrast, the same loop in *Mtub*MetRS is rotated by $\sim 90^\circ$, adopting a conformation which brings the motif into proximity to the adenosine ring of Met-AMP. This is an intermediate position when compared with the structure of *Leishmania major* MetRS (*Lmaj*MetRS)

in complex with Met-AMP and pyrophosphate (PDB entry 3kfl; Larson *et al.*, 2011), in which the KMSKS loop adopts a closed conformation towards the active site as a result of an $\sim 30^\circ$ movement of the loop further towards the Met-AMP-binding pocket. The loop in human cytosolic apo MetRS (*Hsap*CytoMetRS) adopts essentially the same conformation as that in *Mtub*MetRS. Based on the various positions of the KMSKS loop in these structures it is not easy to find a correlation between the ligands bound and the conformation of the KMSKS loop. For instance, the presence of Met-AMP in both the *L. major* and *M. tuberculosis* enzymes resulted in intermediate and closed KMSKS loop conformations, respectively. Amino-acid sequence differences and crystallization conditions might obviously also play a role in determining which conformation the flexible KMSKS loop adopts.

Regarding tRNA^{Met} binding, the mainly helical ABD of *Mtub*MetRS superimposes with an r.m.s.d. of 1.00 Å for the C α atoms onto the equivalent domain of *Aquifex aeolicus* MetRS (*Aaeo*MetRS) in complex with tRNA^{Met} (PDB entry 2ct8; Nakanishi *et al.*, 2005). Four out of the five residues, Asn353, Arg357, Trp422, Leu491 and Phe492, that are involved in the recognition of the anticodon triplet $^{34}\text{CAU}^{36}$ of tRNA^{Met} in *Aaeo*MetRS are conserved in *Mtub*MetRS (Asn357, Arg361, Trp431, Val506 and Phe507, respectively), while Leu491 of the mycobacterial enzyme is very similar to Val506 of *Aaeo*MetRS. Therefore, tRNA^{Met} anticodon recognition by *Mtub*MetRS is expected to be similar to that by *Aaeo*MetRS (Fig. 3).

3.2. Interactions of *Mtub*MetRS with Met-AMP

Methionine and ATP were essential for obtaining *Mtub*MetRS crystals. Their reaction led to the formation of Met-AMP, which was bound to the active site of the enzyme, as seen in the electron-density map (Fig. 4a). Two pockets can

Figure 1
Domain organization of *Mtub*MetRS. The Rossmann-fold catalytic domain (CD) is shown in blue with the connective peptide domain (CP) in green. CP subdomains CP1 and CP2 are labeled. The stem-contact fold domain (SCF) containing the KMSKS loop is shown in yellow. The π -helix connecting the SCF and the anticodon-binding domain (ABD) is depicted in cyan. The ABD is depicted in red. Methionyl adenylate (Met-AMP) is shown as spheres with C atoms in pink, N atoms in blue, O atoms in red, P atoms in orange and S atoms in yellow.

Figure 2
Variability of KMSKS loop conformations among MetRS structures. The *Mtub*MetRS•Met-AMP structure is shown in blue, that of *Lmaj*MetRS•Met-AMP•PP_i (PDB entry 3kfl; Larson *et al.*, 2011) is in gray, *Msm*MetRS•Met (PDB entry 2x1m; Ingvarsson & Unger, 2010) is in brown and *Msm*MetRS•Met•adenosine (PDB entry 2x1l; Ingvarsson & Unger, 2010) is in cyan. Methionyl adenylate (Met-AMP) bound to *Mtub*MetRS is depicted with C atoms in pink, N atoms in blue, O atoms in red, P atoms in orange and S atoms in yellow.

be defined in the active site: the methionine-binding pocket (MBP), where the methionyl group of Met-AMP is situated, and the adenine-binding pocket (ABP), where the adenine moiety of Met-AMP binds (Fig. 4b).

Fig. 5 summarizes the *MtubMetRS* residues binding to the methionyl, phosphoryl, ribosyl and adenylyl groups of Met-AMP, along with a sequence alignment with the MetRSs from other species mentioned in this paper. Most of the interactions between the Met-AMP methionyl group and the enzyme in the MBP are hydrophobic and are mediated by Ala9, Trp228, Ala231, Leu232, Tyr235, Ile264 and His268. The N atom of the methionyl moiety makes hydrogen bonds to the carbonyl of Ile10 and to the side-chain carboxylate of Asp49. One O atom of the phosphoryl group of Met-AMP forms hydrogen bonds to the main-chain amino group of Tyr12 as well as to the imidazole ring of His21. The *MtubMetRS* residues that form hydrogen bonds to the ribose ring are Glu24, Gly261 and Asp263. Specifically, the 3'-hydroxyl group of the ribose is engaged in hydrogen bonds to the carboxylate of Glu24 and to the main-chain amino group of Gly261. At the same time, the 2'-hydroxyl hydrogen-bonds to the carboxylate of Asp263. In the ABP, the N₇ atom of the Met-AMP adenine moiety interacts with the imidazole ring of residue His18, while the N₁ and N₆ atoms are engaged in hydrogen bonds to the Leu293 main-chain carbonyl and amino groups, respectively. Addi-

tionally, Gly20 and Phe292 make hydrophobic contacts with the Met-AMP adenine ring in the ABP.

In summary, extensive interactions, both hydrophobic and hydrophilic (Fig. 4c), mediate the formation of the *MtubMetRS*·Met-AMP complex, with a buried surface area of 919 Å² as calculated by PISA (Krissinel & Henrick, 2007).

Fig. 6 shows the temperature-factor (*B*-factor) distribution for the Met-AMP molecule in the crystal. The methionyl moiety has a lower *B* factor than the adenosine group of Met-AMP. This might be owing to the fact that the methionyl end of the Met-AMP molecule is better anchored to the protein

Figure 3

Comparison of the anticodon-binding domains (ABDs) of *A. aeolicus* MetRS (*AaeoMetRS*) and *MtubMetRS*. *MtubMetRS*·Met-AMP is in blue and *AaeoMetRS*·Met-AMP·tRNA^{Met} (PDB entry 2ct8; Nakanishi *et al.*, 2005) is in light green (protein) and yellow (tRNA). Residues involved in the binding of *AaeoMetRS* to tRNA^{Met} and the anticodon triplet 3⁴CAU³⁶ are shown as sticks and labeled. C atoms are colored green (*AaeoMetRS*) or yellow (tRNA^{Met}), N atoms blue and O atoms red. By homology, the residues expected to bind tRNA^{Met} in *MtubMetRS* would be Asn357 in helix π 1, Arg361 in helix α 15, Trp431 in helix α 19 and Val506 and Phe507 at the C-terminal end of *MtubMetRS*. The labels of secondary-structure elements are those for the *MtubMetRS*·Met-AMP crystal structure.

Figure 4

Met-AMP binding to *MtubMetRS*. (a) Methionyl adenylate (Met-AMP) difference electron-density map calculated by omitting the molecule, contoured at 2 σ (positive density in gray, negative density in red). (b) General features of the Met-AMP binding mode. Shown are the protein surface (in light blue), bound Met-AMP as sticks and the two pockets: the methionine-binding pocket (MBP) and the adenine-binding pocket (ABP). (c) The hydrogen-bond network in the *MtubMetRS*·Met-AMP interaction is shown as dotted red lines. *MtubMetRS* residues interacting with Met-AMP are labeled. Met-AMP C atoms are shown in pink, N atoms in blue, O atoms in red, P atoms in orange and S atoms in yellow.

Res. No.	9	10	11	12	18	20	21	24	49	51	52	53	54	211	214	215	224	225	226	227	228	230	231	232	234	235	261	263	264	267	268	292	293	OI%	MI%	II%	
Met-AMP-binding pocket	MBP	MBP		MBP P	ABP	ABP	P	R	MBP												MBP		MBP	MBP		MBP	R	R	MBP		MBP	ABP	ABP				
Inhibitor-binding pocket	EMP	EMP	EMP	LR					AP	AP	AP	AP	AP	AP	AP	AP	AP	AP	AP	AP	EMP	AP	EMP	EMP	EMP	EMP			EMP	EMP	EMP						
<i>M. tuberculosis</i>	A	I	A	Y	H	G	H	E	D	H	G	L	K	F	G	V	V	M	Y	V	W	D	A	L	N	Y	G	D	I	F	H	F	L	N/A	N/A	N/A	
<i>M. smegmatis</i>	A	I	A	Y	H	G	H	E	D	H	G	Q	K	F	G	V	V	M	Y	V	W	D	A	L	N	Y	G	D	I	F	H	W	L	76	94	96	
<i>B. melitensis</i>	A	I	A	Y	H	G	H	E	D	H	G	I	K	F	G	I	V	M	Y	V	W	D	A	L	N	Y	G	D	I	F	H	F	L	61	100	92	
<i>S. aureus</i>	P	I	Y	Y	H	G	H	S	D	H	G	Q	K	F	G	V	V	V	Y	V	W	D	A	L	N	Y	A	E	I	F	H	W	I	41	67	84	
<i>T. brucei</i>	P	I	Y	Y	H	G	H	S	D	H	G	Q	K	L	A	I	C	V	Y	V	W	D	A	L	N	Y	G	D	I	F	H	W	W	42	78	68	
<i>H. sapiens mito</i>	P	I	F	Y	H	G	H	S	D	H	G	L	K	L	G	I	T	I	Y	V	W	D	A	L	N	Y	G	D	I	F	H	H	W	38	78	76	
<i>H. sapiens cyto</i>	A	L	P	Y	H	G	N	C	D	Y	G	T	A	L	G	T	V	F	Y	V	W	D	A	T	G	Y	A	D	N	F	H	Y	L	26	56	56	

Figure 5
Amino-acid sequence alignment of residues forming the Met-AMP-binding and inhibitor-binding pockets in different species. Met-AMP-binding pocket: MBP, methionine-binding pocket; ABP, adenine-binding pocket; P, Met-AMP phosphate-binding site; R, Met-AMP ribose-binding site. Inhibitor-binding pocket: EMP, enlarged methionine pocket; LR, linker region; AP, auxiliary pocket. Residues indicated as part of the inhibitor-binding pocket are predicted to bind the MetRS inhibitors based on previous MetRS-inhibitor crystal structures [for example, *Tbru*MetRS (Huang *et al.*, 2016, 2017; Koh *et al.*, 2012, 2014; Zhang *et al.*, 2016), *Bmel*MetRS (Ojo *et al.*, 2016) and *Saur*MetRS (PDB entries 4qrd and 4qre)]. OI, percentage overall protein sequence identity; MI, percentage identity for Met-AMP-binding pocket residues; II, percentage identity for inhibitor-binding pocket residues.

than the adenosine end. This idea corresponds with the fact, discussed below, that the side chain of Phe292 near the adenine ring has the highest *B* factor in the *Mtub*MetRS Met-AMP binding site (Fig. 6). Alternatively, it is possible that Met-AMP is not present at full occupancy, but that instead the *Mtub*MetRS crystal used contains a mixture of *Mtub*MetRS-Met-AMP and *Mtub*MetRS-Met complexes (possibly even with partial occupancy). This might be somewhat similar to the structure of *Msm*MetRS-Met-adenosine (PDB entry 2x1l; Ingvarsson & Unge, 2010), where the adenosine molecule presents *B* factors that are more than three times higher than those of the methionine molecule.

3.3. The Met-AMP-binding pocket among methionyl-tRNA synthetases

There are a considerable number of MetRS structures with an adenine ring occupying the adenine-binding pocket. Here, we compare adenine binding in a number of pathogen structures and the only human MetRS structure to date: that of the human cytosolic enzyme. Fig. 5 shows the sequence alignment of *Mtub*MetRS with *Msm*MetRS (its closest homolog with known structure; Ingvarsson & Unge, 2010) and additional selected MetRS enzymes. Phe292 is the only residue interacting directly with Met-AMP that differs in the two mycobacterial enzymes. The side chain of Phe292 in *Mtub*MetRS exhibits an intriguing difference in conformation with respect to the equivalent Trp294 in *Msm*MetRS. While the indole ring of Trp294 adopts an inward conformation (‘in’) in *Msm*MetRS-Met-adenosine, resulting in a stacking hydrophobic interaction with the adenine ring (Fig. 7b) of the adenosine molecule (PDB entry 2x1l; Ingvarsson & Unge, 2010), the phenyl side-chain group of Phe292 is in an ‘out’ conformation in *Mtub*MetRS-Met-AMP, with a single atom of Phe292 in contact with the adenine ring at a distance of 3.9 Å. The high *B* factor of the Phe292 side chain (Figs. 6 and 7a) suggests that the side chain of Phe292 adopts multiple conformations. In the structure of *Tbru*MetRS-Met-AMP

(PDB entry 4eg3; Koh *et al.*, 2012), the equivalent Trp547 interacts with the adenine ring of Met-AMP with an ‘in’ conformation (Fig. 7c). In the structure of a truncated version of *Hsap*CytoMetRS, comprising residues 221–834, the residue corresponding to Phe292 in *Mtub*MetRS is Tyr586 and its side chain adopts an ‘out’ conformation (Fig. 7d). In several MetRS structures with a filled adenine pocket, a homologous residue corresponding to *Mtub*MetRS Phe292 is present, which in some instances is in an ‘in’ conformation (for example in *Aaeo*MetRS-Met-AMP-tRNA; PDB entry 2ct8) and in other cases is in an ‘out’ conformation (for example in *Lmaj*MetRS-Met-AMP-PP_i; PDB entry 3kfl). Hence, the conformation that the residue equivalent to Phe292 in *Mtub*MetRS adopts in related MetRSs is clearly highly vari-

Figure 6
Temperature factors of Met-AMP and the catalytic pocket. The *Mtub*MetRS catalytic pocket and Met-AMP are shown colored according to *B* factors, with cyan corresponding to the lowest and red to the highest values. Of the residues binding Met-AMP in the *Mtub*MetRS-Met-AMP structure, Phe292 has the highest *B* factor (around 100 Å²), which corresponds to the high *B* factors of the adenosyl group of the Met-AMP molecule.

able. The human mitochondrial enzyme presents residue His340 in the same position and it is obviously hard to predict the conformation that this residue could adopt. Nevertheless, the structural and sequence differences near the adenine-binding pocket of *MtubMetRS* versus the human homologs might be of help in the future development of antituberculosis chemotherapeutics.

3.4. *M. tuberculosis* MetRS versus its human homologs

It is imperative to compare the *MtubMetRS* structure with those of its human homologs to evaluate the potential for arriving at selective inhibitors. Superposition of *MtubMetRS* onto the available human cytosolic apo MetRS (*HsapCytoMetRS*) crystal structure (PDB entry 5gl7; H. Y. Cho, H. J. Lee & B. S. Kang, unpublished work) yields an overall r.m.s.d. of 1.26 Å for the C α atoms. The C α atoms of the catalytic and

anticodon-binding domains of these two structures superpose quite well, with r.m.s.d. values of 1.07 and 1.11 Å, respectively. When superposing the catalytic domains of the two structures, the connective peptide CP1 subdomain of *MtubMetRS* is moved considerably 'downwards' towards the active site, adopting a closed conformation (Fig. 8a) as described before for *MsmMetRS* (Ingvarsson & Unge, 2010), differing from the conformation of CP1 in human cytosolic MetRS and in other MetRSs (Serre *et al.*, 2001; Crepin *et al.*, 2004). Especially interesting is the fact that the same subdomain changes conformation upon the binding of inhibitors in *TbruMetRS* (Koh *et al.*, 2012, 2014; Zhang *et al.*, 2016; Huang *et al.*, 2016, 2017). In the *TbruMetRS*·Met-AMP structure, CP1 is in the closed conformation, similar to that described here for *MtubMetRS*·Met-AMP, but it adopts an open conformation when a *TbruMetRS* inhibitor is bound (Koh *et al.*, 2012). In the case of the human apo cytosolic enzyme structure, CP1 is

Figure 7

Interactions of Phe292 of *MtubMetRS* with the Met-AMP adenine ring and comparison with selected MetRS structures. (a) Met-AMP adenine-ring interaction with Phe292 (sticks) in the *MtubMetRS*·Met-AMP structure. *MtubMetRS* is shown in blue with the C atoms of Met-AMP in pink. (b) In the *MsmMetRS*·Met-adenosine structure (PDB entry 2x1l; Ingvarsson & Unge, 2010) the adenine ring of adenosine engages in a hydrophobic stacking interaction with Trp294, which is equivalent to Phe292 of *MtubMetRS*. Trp294 is shown as sticks. *MsmMetRS* is colored cyan, with methionine C atoms in gray and adenosine C atoms in purple. (c) Likewise, in the *TbruMetRS*·Met-AMP structure (PDB entry 4eg3; Koh *et al.*, 2012) the adenine ring of Met-AMP engages in a hydrophobic stacking interaction with Trp547 (shown as sticks; equivalent to Phe292 of *MtubMetRS*). *TbruMetRS* is colored green with Met-AMP C atoms in pink, N atoms in blue, O atoms in red, P atoms in orange and S atoms in yellow. (d) Surface representation of the Met-AMP-binding pocket of the apo *HsapCytoMetRS* (PDB entry 5gl7) structure. *HsapCytoMetRS* is shown in orange. Tyr586, which is equivalent to Phe292 in *MtubMetRS*, is depicted as sticks. The empty MBP and ABP pockets are labeled.

in a much more open conformation (Fig. 8*a*). The structural differences regarding CP1 might be exploitable when aiming for selectivity of compounds against *Mtub*MetRS, since CP1 residues could potentially interact with a compound designed to bind specifically to the mycobacterial enzyme, keeping the CP1 knuckle in the open conformation, and thereby prevent catalytic action.

Overall, *Mtub*MetRS and its human mitochondrial (*Hsap*-MitoMetRS) and cytosolic counterparts are 38 and 26% identical in sequence, respectively, with 78 and 56% identity,

respectively, when comparing residues binding Met-AMP in *Mtub*MetRS with those in *Hsap*MitoMetRS and *Hsap*-CytoMetRS (Fig. 5). When comparing the available *Hsap*-CytoMetRS structure with that of *Mtub*MetRS, there is a significant difference between the two enzymes close to the Met-AMP ribose-binding site in *Mtub*MetRS. The homologous segment to helix $\alpha 1$ of *Mtub*MetRS forms two helices in *Hsap*CytoMetRS, $\alpha 2$ and $\alpha 3$ (Figs. 8*b*, 8*c* and 9), separated by a hinge region. This secondary-structure difference is accompanied by the presence of a 'human cytosolic extra

Figure 8

Comparison of the *Mtub*MetRS and *Hsap*CytoMetRS structures. (a) Superposition of the *Hsap*CytoMetRS (PDB entry 5gl7; light orange) and *Mtub*MetRS (blue) catalytic domains. The mainly β subdomain CP1 of *Mtub*MetRS is shifted 'downwards' towards the active site (black arrow) compared with the human enzyme. (b) In the catalytic domain, a distinct difference between the conformation of *Mtub*MetRS $\alpha 1$ and that of the same region of *Hsap*CytoMetRS is evident. (c) Close to where the Met-AMP ribose ring binds in *Mtub*MetRS, instead of a single helix $\alpha 1$, *Hsap*CytoMetRS has two helices $\alpha 2$ and $\alpha 3$ connected by a hinge. (d) A pocket, called the human cytosolic extra pocket (HCEP), harboring four waters (red spheres) is present in *Hsap*CytoMetRS close to the ribose-binding region (ribose not shown). (e) The presence of His21 makes this pocket smaller in *Mtub*MetRS, containing only one water molecule. Met-AMP C atoms are colored pink, N atoms blue, O atoms red, P atoms orange and S atoms yellow.

Figure 9

Sequence alignment of the *MtubMetRS* α 1-helix region with those of human MetRS homologs. In *HsapCytoMetRS* the secondary structure of the region corresponds to two α -helices: α 2 and α 3. The insertion, compared with both *MtubMetRS* and *HsapMitoMetRS*, of Gly286 in *HsapCytoMetRS* is indicated by a red box. Conserved residues are shown on a black background, conservative changes on a gray background and divergent residues on a white background.

pocket' (HCEP) harboring four water molecules in *HsapCytoMetRS* (Fig. 8d). Owing to both the difference in the secondary structure of helix α 1 of *MtubMetRS* versus helices α 2 and α 3 in *HsapCytoMetRS*, as well as the positioning of the pocket-filling side chain of residue His21 in *MtubMetRS*, the bacterial pocket is smaller, harboring one water molecule instead of four (Fig. 8e). When aligning the bacterial and human sequences, the reason for this difference appears to be the insertion of an extra glycine, Gly286, in the human cytosolic protein, exactly at the connection between the helices (Fig. 9). In the case of *HsapMitoMetRS*, the structure of which is not available, the amino-acid sequence lacks the extra glycine residue present in *HsapCytoMetRS* between helices α 2 and α 3. Therefore, the hinge would be expected to be absent, probably resulting in a secondary structure similar to that of helix α 1 in *MtubMetRS* and the concomitant absence of the HCEP pocket in the human mitochondrial enzyme.

Regarding the design of compounds that have higher affinity for tuberculosis MetRS than for the human homologs, it is of interest that in the Met-AMP-binding region of the human cytosolic enzyme one in about every two residues differs from that in *MtubMetRS* (Fig. 5). Moreover, the cytosolic enzyme is a MetRS2 enzyme and *MtubMetRS* is a MetRS1 enzyme. As reported before for *SaurMetRS*, many compounds inhibiting bacterial MetRS1 are not potent against the human cytosolic enzyme, which belongs to the MetRS2 form (Green *et al.*, 2009). Specifically, REP8839 was reported to have a selectivity of more than 1 000 000-fold for *SaurMetRS* over the 27% identical human cytosolic enzyme (Green *et al.*, 2009). Therefore, differentially targeting *MtubMetRS* and not the cytosolic enzyme should be readily feasible.

This is somewhat different for the human mitochondrial enzyme since the *M. tuberculosis* and human mitochondrial enzymes both belong to the MetRS1 form. Nevertheless, 22% of the residues forming the Met-AMP-binding site in *M. tuberculosis* and human mitochondrial MetRS are different, and 24% in the inhibitor-binding site (Fig. 5). Moreover, compound REP8839 mentioned above has a 1000-fold higher affinity for the *S. aureus* enzyme than for human mitochondrial MetRS (Green *et al.*, 2009), while the amino-acid difference in the inhibitor-binding site between the *S. aureus* and human mitochondrial enzymes is 24%, *i.e.* the same as in the comparison of *M. tuberculosis* and human mitochondrial MetRS. The fact that considerable selectivity

between human mitochondrial and bacterial MetRS enzymes could be achieved in the case of *S. aureus* is promising and indicates that there are opportunities to arrive at inhibitors that have a higher affinity for the *M. tuberculosis* MetRS than for both human homologs.

In conclusion, this paper reports the crystal structure of *M. tuberculosis* MetRS bound to its substrate intermediate. Analysis of the structure reveals differences from the human orthologs that will be useful for designing selective inhibitors in the effort to discover new antituberculosis drugs.

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